Interactions between glucocorticoid metabolism and inflammation in obesity and insulin resistance.

Mark Nixon

Doctor of Philosophy

The University of Edinburgh

2011

Declaration

I declare that this thesis was written by me and that the data presented within it is a result of my own work performed at The University of Edinburgh, except the procedures listed below.

Surgery for minipump implantation and glucose tolerance tests for 'lean' group of mice in Chapter 3 were performed by Dr Dawn Livingstone of the Centre for Cardiovascular Science, Queen's Medical Research Institute, University of Edinburgh.

I declare that this work has not been previously submitted for any other degree or qualification.

Mark Nixon

Acknowledgments

I would like to thank my supervisors Ruth Andrew, Brian Walker and Adriano Rossi for their fantastic support throughout my PhD studies. Their guidance, encouragement and unwavering enthusiasm have provided a great environment in which to work.

I am indebted to numerous people within The Queen's Medical Research Institute for their help and advice. I would like to thank all those within Endocrinology, in particular the members of the 'A-team', Dawn Livingstone, Kerry McInnes and Carolynn Cairns, as well as Tiina Kipari and Janet Man-Erskine, all of whom without which I would have been completely lost. I would like to thank all those, both past and present members, in the C3.01 office, for putting up with me for over three years (although being the only man in the office for almost two years, I still maintain I deserve a medal for putting up with them!). Also, I would like to say special thanks to some of the other PhD students who have helped make my time both in and out of the lab so enjoyable, particularly Rachel Dakin, Lucy Low, Yvonne Nelson and Tijana Mitic. In the CIR, I'm extremely grateful to Rodger Duffin for all his advice and support with all things inflammation-related.

I would also like to thank all the staff in the BRF for their help with all animal-related issues, in particular Keith and Duncan for making long afternoons more entertaining with their 'diamond banter.'

Finally, and perhaps most importantly, I would like to thank my family. My parents David and Audrey and my brother Philip have given me amazing support throughout everything I've done, as well as always having the best cooked food in the land for me whenever I made it home to Northern Ireland. Their belief in me has been an immense source of strength throughout my PhD and I'm forever indebted to them for pushing me to achieve greater things.

‘IF you can keep your head when all about you
Are losing theirs and blaming it on you,
If you can trust yourself when all men doubt you,
But make allowance for their doubting too;
If you can wait and not be tired by waiting,
Or being lied about, don't deal in lies,
Or being hated, don't give way to hating,
And yet don't look too good, nor talk too wise:

If you can dream - and not make dreams your master;
If you can think - and not make thoughts your aim;
If you can meet with Triumph and Disaster
And treat those two impostors just the same;
If you can bear to hear the truth you've spoken
Twisted by knaves to make a trap for fools,
Or watch the things you gave your life to, broken,
And stoop and build 'em up with worn-out tools:

If you can make one heap of all your winnings
And risk it on one turn of pitch-and-toss,
And lose, and start again at your beginnings
And never breathe a word about your loss;
If you can force your heart and nerve and sinew
To serve your turn long after they are gone,
And so hold on when there is nothing in you
Except the Will which says to them: 'Hold on!'

If you can talk with crowds and keep your virtue,
' Or walk with Kings - nor lose the common touch,
if neither foes nor loving friends can hurt you,
If all men count with you, but none too much;
If you can fill the unforgiving minute
With sixty seconds' worth of distance run,
Yours is the Earth and everything that's in it,
And - which is more - you'll be a Man, my son!’

Rudyard Kipling

Contents

Abstract

Inflammation plays a key role in the underlying pathogenesis of obesity and its associated health risks, with increased markers of inflammation evident in both liver and adipose tissue. In parallel, there is dysregulation of glucocorticoid metabolism in obesity, with increased adipose levels of the glucocorticoid-regenerating enzyme 11β-hydroxysteroid dehydrogenase type 1 (11βHSD1) and increased hepatic levels of 5α-reductase type 1 (5αR1), which catalyses the reduction of glucocorticoids. Both the mechanisms and consequences of this glucocorticoid metabolism dysregulation remain unclear, however, there is evidence that it may be related to inflammation. *In vitro* studies have demonstrated that pro-inflammatory markers upregulate 11βHSD1 expression in adipocytes, potentially explaining increased expression of this enzyme in obesity. Previous work has also demonstrated that the glucocorticoid metabolites produced by 5αR1 lack the metabolic effects of the parent glucocorticoid, but retain its anti-inflammatory properties, indicating that increased expression of hepatic 5αR1 may serve to dampen down inflammation in the liver. The hypotheses addressed in this thesis are that in obesity, inflammation regulates adipose glucocorticoid metabolism through 11βHSD1, and that hepatic glucocorticoid metabolism regulates the inflammatory state of the liver through 5αR1.

The role of inflammation in the regulation of 11βHSD1 was assessed *in vivo* in mice treated with the anti-inflammatory compound sodium salicylate (salicylate). In diet-induced obese mice, salicylate downregulated 11βHSD1 expression and activity selectively in visceral adipose tissue, alongside improved glucose tolerance, reduced plasma non-esterified fatty acids, and changes in adipose lipid metabolism. 11βHSD1-deficient mice fed a high-fat diet were resistant to the insulin sensitising effects of salicylate treatment. These results indicate a novel role for 11βHSD1 down-regulation in mediating the insulin sensitising effect of anti-inflammatory treatment.

The mechanisms underpinning the anti-inflammatory properties of 5α-reduced glucocorticoids were explored *in vitro* and *in vivo*. In lipopolysaccharide-stimulated murine macrophages, both 5α-reduced glucocorticoid metabolites tested, namely 5α-dihydrocorticosterone (5αDHB) and 5α-tetrahydrocorticosterone (5αTHB), suppressed tumor necrosis factor-α (TNFα) and interleukin-6 (IL-6) release, although to a lesser extent than corticosterone (B). Similar to B, both 5αDHB and 5α THB suppressed phosphorylation of intra-cellular inflammatory signalling mitogen-activated protein kinases (MAPK) proteins c-Jun N-terminal kinase (JNK) and p38, as well as increasing protein expression of MAPK phosphatase-1 (MKP-1). Treatment of phorbol ester-stimulated HEK293 kidney cells with these 5α-metabolites revealed that 5αDHB suppressed nuclear factor κB (NFκB) and activator protein-1 (AP-1) activation to a similar extent to that of B, whilst 5αTHB increased activation of these pro-inflammatory transcription factors, indicating cell-specific effects of 5αTHB.

In conclusion, reduced intra-adipose glucocorticoid regeneration by 11βHSD1 mediates the insulin sensitising effects of salicylate, suggesting that altered glucocorticoid metabolism may reflect altered intra-adipose inflammation in obesity. Furthermore, these data support the concept that this enzyme provides a therapeutic target in obesity-related metabolic disorders. 5α-reduced metabolites of glucocorticoids have similar anti-inflammatory properties to the parent glucocorticoid, indicating that the elevated hepatic levels of 5α-reductase in obesity may be a protective mechanism to limit the adverse metabolic effects of glucocorticoids upon the liver, but maintain the beneficial anti-inflammatory properties. These 5α-reduced glucocorticoid metabolites may provide a potential therapeutic treatment as selective glucocorticoid receptor modulators for inflammatory conditions.

List of Figures

List of Tables

Abbreviations

11βHSD	11β-hydroxysteroid dehydrogenase
3αHSD	3α-hydroxysteroid dehydrogenase
5αDHB	5α-dihydro corticosterone
5αR	5α-reductase
5αTHB	5α-tetrahydro corticosterone
5βR	5β-reductase
ACTH	Adrenocorticotropic hormone
AdiQ	Adiponectin
AF	Activation function
Agt	Angiotensinogen
AKR	Aldo-keto reductase
AP	Activator protein
ATGL	Adipose triglyceride lipase
B	Corticosterone
β-HB	β-hydroxybutyrate
bp	Base pair

BSA	Bovine serum albumin
cDNA	Complementary DNA
CMV	Cytomegalovirus
Cp	Crossing point
CRH	Corticotrophin-releasing hormone
DEPC	Diethylpyrocarbonate
DHC	Dehydrocorticosterone
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-regulated kinase
FDPase	Fructose-1,6-diphosphatase
FITC	Fluorescein isothiocyanate
G6Pase	Glucose-6-phosphatase
GABA	Gamma-aminobutyric acid

GK	Glycerol kinase
GLUT	Glucose transporter
GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
GTT	Glucose tolerance test
h	Hour
HAT	Histone acetyl transferase
HDAC	Histone deacetylase
HEK293	Human embryonic kidney cell line
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
Hop	Hsp organizing protein
HSL	Hormone sensitive lipase
Hsp	Heat shock protein
ICAM	Inter-cellular adhesion molecule
i.p.	Intraperitoneal
IL	Interleukin
IκB	Inhibitor of nuclear factor kappa-B

IKK	Inhibitor of nuclear factor kappa-B kinase
IRS	Insulin receptor substrate
JNK	c-Jun N-terminal kinase
KO	Knock-out
LB	Luria Bertani
LPL	Lipoprotein lipase
LPS	Lipopolysaccharide
Luc	Luciferase
MAPK	Mitogen-activated protein kinase
MCP	Monocyte chemoattractant protein
MKP	MAPK phosphatase
MMTV	Mouse mammary tumour virus
MR	Mineralocorticoid receptor
MW	Molecular weight
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NCoR	Nuclear receptor corepressor

NEFA	Non-esterified fatty acid
NFκB	Nuclear factor-kappa B
OFN	Oxygen-free nitrogen
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PEPCK	Phosphoenolpyruvate carboxykinase
PerCP/Cy5.5	Peridinin chlorophyll protein complex/Cy5.5
PMNT	Phenylethanolamine-N-Methyl Transferase
POMC	Pro-opiomelanocortin
RAW264.7	Murine macrophage cell line
RT-PCR	Reverse transcriptase polymerase chain reaction
SGRM	Selective glucocorticoid receptor modulator
SMRT	Silencing mediator of retinoic acid and thyroid hormone receptor
SPA	Scintillation proximity assay
SRC	Steroid receptor coactivator
STAT	Signal transducer and activator of transcription

SV	Simian virus
TAT	Tyrosine aminotransferase
TBS	Tris-buffered saline
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TPA	12-O-Tetradecanoyl-phorbol-13-acetate
Tris	Trisma base
TF	Transcription factor
WT	Wild type

List of Publications

Abstracts

Nixon M, Yang CA, Duffin R, Rossi AG, Walker BR and Andrew R (2011). Anti-inflammatory properties of 5α-Reduced Glucocorticoids: Potential Dissociated Steroids. *Endocrine Abstracts 22, 0303.*

Nixon M, Duffin R, Rossi AG, Walker BR and Andrew R (2010). Anti-inflammatory properties of 5α-Reduced Glucocorticoids: Potential Dissociated Steroids. *Program of Scottish Society for Experimental Medicine 2010.*

Nixon M, Livingstone DEW, Andrew R, and Walker BR. (2010). Salicylate-induced insulin sensitisation in obesity is mediated through downregulation of omental adipose 11-hydroxysteroid dehydrogenase type 1. *Program of the 92nd Meeting of the Endocrine Society, OR30-6.*

Nixon M, Livingstone DEW, Andrew, R, and Walker BR. (2010). Down-regulation of adipose 11βHSD1 mediates the insulin sensitising effects of salicylate in obesity. *CRF Conference 2010 Diabetes and Obesity, Royal Society of Edinburgh.*

Nixon M, Livingstone DEW, Andrew, R, and Walker BR. (2010). Down-regulation of adipose 11βHSD1 mediates the insulin sensitising effects of salicylate in obesity. *Endocrine Abstracts 21, P187.*

Nixon M, Livingstone DEW, Andrew R, and Walker BR. (2009). Down-regulation of omental adipose 11β-hydroxysteroid dehydrogenase type 1 may mediate the insulin sensitising effects of salicylates in obesity. *Program of Scottish Society for Experimental Medicine 2009.*

Chapter 1

Introduction

The prevalence of obesity continues to escalate in the western world, with the condition also becoming an increasing problem in developing countries. Projections by the World Health Organisation indicate that by 2015, approximately 2.3 billion people will be overweight, with more than 700 million obese. With this rise in obesity comes an increased incidence of obesity-related disorders, including non-alcoholic fatty liver disease (NAFLD), type 2 diabetes and cardiovascular disease. There is increasing evidence that obesity is associated with low-grade inflammation, as well as a dysregulation in glucocorticoid metabolism. This chapter will introduce the background to the inflammation that is apparent in obesity, as well the roles played by glucocorticoids and their metabolism.

1.1 Inflammation in obesity

In the past decade, it has become clear that inflammation plays a critical role in the metabolic dysfunction associated with obesity, namely the development of insulin resistance and progression through to type 2 diabetes (Chen 2006). The concept for a role of inflammation in obesity first appeared when it was demonstrated that expression of the pro-inflammatory cytokine tumor necrosis factor-α (TNFα) was increased in obese animals, in both the circulation and the adipose tissue (Hotamisligil *et al.* 1993). Further studies found that, in several obese mouse models, functional TNFα-deficiency improved insulin sensitivity (Uysal *et al.* 1997). Such observations were also evident in humans, with increased adipose tissue expression of TNFα in obese subjects, followed by a drop in expression after weight loss (Hotamisligil *et al.* 1995; Dandona *et al.* 1998). This work also raised the possibility that other pro-inflammatory molecules aside from TNFα contribute to the development of insulin resistance. Indeed, interleukin-6 (IL-6), another pro-inflammatory molecule, was implicated in obesity-related disorders soon after TNFα (Bastard *et al.* 2002; Sabio *et al.* 2008). In rodent genetic models of obesity, anti-IL-6 treatment improves insulin sensitivity (Klover *et al.* 2005), whilst in humans, circulating levels of IL-6 decrease with weight loss and improvement in insulin sensitivity (Kopp *et al.* 2003; Monzillo *et al.* 2003).

1.1.2 Role of immune cells in the origin of inflammation in obesity

Given their increased levels in obesity, intense work has gone into establishing the origin of pro-inflammatory cytokines. Whilst it is now recognised that they are released by numerous cell types (Maury and Brichard 2010), the primary source of inflammatory mediators within the body remains cells of the immune system, including dendritic cells, neutrophils and macrophages (Fain ; Olefsky and Glass 2010). This suggests that in obesity, there is an activation of the host innate immune system, driving an inflammatory response. Inflammation itself is a crucial component of tissue repair and involves numerous distinct cell types. However, prolonged inflammation is often detrimental, as appears to be the case in metabolic diseases. Whilst studies had shown obesity was associated with increased circulating levels of pro-inflammatory molecules (Hotamisligil *et al.* 1993; Hotamisligil *et al.* 1995; Uysal *et al.* 1997; Dandona *et al.* 1998; Bastard *et al.* 2002), it was the discovery of increased inflammation within the liver, and in particular, adipose tissue, that revealed the potential source of the inflammatory response (Chen 2006; Hotamisligil 2006). This crucial 'overlap' in the metabolic and inflammatory pathways may, in part, be explained from an evolutionary viewpoint.

The Drosphilia fat body, which incorporates the mammalian homologues of the liver and haematopoietic and immune systems, is thought to be a common ancestral structure that controls key metabolic and immune systems (Hotamisligil 2006). This fat body acts as the site of co-ordination of responses to pathogens and also senses energy and nutrient availability. In higher mammalian organisms, the liver, adipose tissue and haematopoietic system have specialized into distinct organ systems. However, they have maintained their developmental heritage. This is evident when the cell types present in these organs are considered. In the adipose tissue, adipocytes are found in close proximity to macrophages, whilst in the liver, specialised macrophages, named Kuppfer cells, are present (Chen 2006). This interface between metabolic and immune cells within these tissues provides an environment whereby factors released by immune cells may act locally to exert adverse metabolic effects.

Initially viewed as a passive tissue, functioning only as a site of energy storage, the understanding of adipose as a metabolically active tissue that regulates many processes has paved the way for greater mechanistic insights into obesity-related conditions (Frayn *et al.* 2003; Kershaw and Flier 2004; Chen 2006). Whilst the levels of several pro-inflammatory cytokines are increased in obesity, it was not clear whether this resulted from release from adipocytes or non-fat cells. Over the past number of years, numerous studies have attempted to dissect the mechanisms underlying inflammation within adipose tissue, with several key factors emerging. Perhaps the most exciting discovery was that obese adipose tissue is infiltrated by macrophages and that these infiltrated immune cells are a major source of pro-inflammatory cytokine release (Weisberg *et al.* 2003). Whilst 'resident' macrophages are present within adipose, obese adipose is characterised by a large increase in the number of bone-marrow-derived macrophages. Indeed, some reports in obese rodents and humans have suggested that up to 40% of total adipose cell content consists of macrophage cells, compared to approximately 10% in lean counterparts (Weisberg *et al.* 2006). However, despite the infiltration of these cells, the mechanisms that recruit and retain macrophages in obese adipose remained unclear.

In order to recruit macrophages, it was speculated that there must by a 'signal' to guide them. Considerable evidence has since accumulated that monocyte-chemoattractant protein-1 (MCP-1), derived from adipocytes and resident macrophages, plays a crucial role in macrophage infiltration (Kamei *et al.* 2006; Kanda *et al.* 2006; Weisberg *et al.* 2006). This chemokine plays a key role in the development of inflammatory responses and promotes the recruitment of monocytes into damaged or inflamed tissues. In obese adipose tissue, MCP-1 expression correlates positively with adiposity (Weisberg *et al.* 2006), whilst mice lacking MCP-1 on a high-fat diet have attenuated macrophage accumulation and inflammation in adipose tissue, as well as improved insulin sensitivity (Inouye *et al.* 2007). This suggests that increased MCP-1 promotes the recruitment of macrophages to adipose tissue, where they contribute to the inflammatory response.

However, the mechanism through which both resident and infiltrated macrophages contribute to this cycle of inflammation remained unclear until the discovery that saturated fatty acids bind toll-like receptor 4 (TLR4) (Song *et al.* 2006). TLRs are a family of receptors for pathogen-associated molecular patterns (PAMPs) that have important roles in innate immunity. They recognise components of foreign objects, namely bacterial elements, which distinguish them from the host. In terms of TLR4, lipopolysaccharide is recognised as a component of the cell wall of gram negative bacteria, stimulating macrophage activation and so initiating an immune response. Recent evidence has demonstrated that TLR4 plays an important role in obesity-induced adipose inflammation, with macrophage expression of TLR4 increased in obesity (Song *et al.* 2006). Furthermore, mice with a myeloid cell-specific deletion of TLR4 were protected from high-fat diet-induced insulin resistance, despite being fully obese (Suganami *et al.* 2007; Saberi *et al.* 2009). The ability of saturated fatty acids to act as an endogenous ligand for TLR4 demonstrates a mechanism by which macrophages become activated in the lipid rich environment of adipose tissue, leading to release of numerous pro-inflammatory molecules.

1.1.3 Role of adipose tissue in obesity

1.1.3.1 Adipocyte role in inflammation induction

Clearly, immune cells have an important role to play in obesity as described above. However, despite illustrating the infiltration of these cells into adipose tissue, it remained unclear as to why such an influx was taking place. Important studies have shown that this macrophage infiltration into adipose precedes the development of insulin resistance, identifying it as a causal factor behind insulin resistance, not merely a consequence (Weisberg *et al.* 2003; Xu *et al.* 2003). A recent breakthrough has been the discovery that obese adipose tissue is characterised by adipocyte hypertrophy (Suganami and Ogawa 2010). Whilst this is an essential process in storing extracellular lipids, in the setting of obesity, adipocyte hypertrophy results in the initiation of the inflammatory signalling cascade (Hotamisligil 2006). During

hypertrophy, multiple pathways are activated in the adipocyte that results in activation of inflammatory signalling. These include the activation of the mitogen-activated protein kinase (MAPK) family of proteins, JNK, p38 kinase and ERK, as well as the down-regulation of MAPK-phosphatase-1 (MKP-1) which normally functions to rapidly inactivate the MAPKs (Johnson and Lapadat 2002; Bost *et al.* 2005). Adipocyte hypertrophy also results in the induction of endoplasmic reticulum (ER) stress and increased expression of reactive oxygen species (Ozcan *et al.* 2004). Both these pathways increase inflammatory signalling within adipocytes. Hypertrophied adipocytes secrete MCP-1 due to their 'inflammatory' state, potentially explaining the source of the increase of this chemokine seen in obesity (Ito *et al.* 2007). Further evidence supporting the idea that hypertrophied adipocytes are the primary 'attraction' for infiltrating macrophages is demonstrated by the apparent 'niche' within the environment of obese adipose tissue that is involved in macrophage activation. Studies have shown that approximately 90% of infiltrated macrophages accumulate around dead adipocytes, forming characteristic structures known as crown-like structures (CLS) (Cancello *et al.* 2005; Cinti *et al.* 2005). These structures form as macrophages surround dead or dying adipocytes, reabsorbing the lipid remnants of these cells.

Whilst the release of pro-inflammatory cytokines from within adipose is due mainly to non-fat cells such as macrophages, adipocytes themselves secrete an array of factors, collectively termed adipokines (Maury and Brichard 2010). These adipokines regulate energy homeostasis and insulin sensitivity in an autocrine, paracrine and endocrine manner, in particular mediating effects in insulin target tissues including the liver and muscle. One of the main adipokines is adiponectin. Unlike most factors released from adipose tissue, the levels of adiponectin are decreased in obesity, such that there is an inverse correlation of adiponectin levels with the development of insulin resistance (Arita *et al.* 1999; Brichard *et al.* 2003). This adipokine is considered an anti-inflammatory factor, which has been shown to reduce weight and improve insulin sensitivity (Kadowaki *et al.* 2006). Both *in vitro* and *in vivo* studies have demonstrated that insulin downregulates adiponectin secretion in adipocytes

(Fasshauer *et al.* 2002; Yu *et al.* 2002), and that insulin sensitising agents markedly increase adiponectin levels (Yu *et al.* 2002). Furthermore, the anti-inflammatory properties of this adipokine were shown in adiponectin-deficient mice which exhibited increased circulating TNFα levels (Maeda *et al.* 2002). The relationship between these two adipokines has been studied extensively, with the overall finding being that they appear to regulate each other in a negative manner (Abbasi *et al.* 2004). In inflammatory adipocytes, adiponectin suppressed TNFα release (Ajuwon and Spurlock 2005), whilst in humans, TNFα has been shown to reduce adiponectin levels in adipose (Hector *et al.* 2007). A reduction in adiponectin expression in obesity would further serve to fuel the inflammatory cycle within adipose, yet adiponectin also has effects in other tissues. In the liver, adiponectin prevents lipid accumulation by increasing β-oxidation and suppressing *de novo* lipogenesis (You *et al.* 2005). Mouse models of liver fibrosis treated with adiponectin attenuates the condition, indicating a protective effect of this adipokine (Kamada *et al.* 2008). This is of particular importance when one considers the condition of the liver in the metabolic syndrome. NAFLD is the hepatic manifestation of obesity-induced metabolic dysfunction, characterised mainly by the excessive hepatic deposition of non-esterified fatty acids (NEFAs) and triglycerides. However, over recent years, evidence has accumulated that pro-inflammatory cytokines contribute to the development of NAFLD, and that these cytokines are derived from adipose tissue (Tsochatzis *et al.* 2009). Increased expression of TNFα has been shown in several studies investigating patients with NAFLD (Valenti *et al.* 2002; Poniachik *et al.* 2006). The role of IL-6 is less clear, with contrasting reports on its expression levels in NAFLD (Tsochatzis *et al.* 2009). However, it appears that inflammation within the liver plays a role in the underlying pathogenesis of obesity-related liver disorders.

1.1.3.2 Differential effects of adipose depots

Whilst obesity is the underlying risk factor for the development of conditions such as NAFLD and type 2 diabetes, it is not generalised obesity *per se*, but rather the regional distribution of adipose in the body that is more closely associated with such metabolic disorders (Bjorntorp 1992; Wajchenberg 2000). Upper body, visceral

adiposity is positively correlated with metabolic dysfunction, as opposed to lower body, subcutaneous (S.C.) adipose. Numerous studies have found that, independent of body mass index, waist circumference (a marker of visceral adiposity) was strongly associated with the prevalence of type 2 diabetes (Garg 2004; Alberti *et al.* 2005; Klein *et al.* 2007). The reason for this lies in the heterogenous nature of adipose tissue, with distinct differences in the biology of adipocytes from visceral adipose and S.C adipose. Indeed, accumulating evidence indicates that S.C adipose may be a 'metabolic sink' that prevents accumulation of detrimental visceral adipose tissue (Sam *et al.* 2008). Given the fact that, even in the obese state, visceral adipose represents a relatively small proportion of total body adipose, it is reasonable to ask why it has such a big influence on whole body metabolism. One possible reason for this is that the venous drainage of visceral adipose is directly into the portal circulation, which in turn drains directly to the liver. As such, the release of factors including NEFAs and pro-inflammatory cytokines from visceral adipose would have direct effects on hepatic metabolism. In support of this, studies have shown that visceral adipose correlates closely with increased liver fat content (Nguyen-Duy *et al.* 2003), and that reductions in visceral adipose are associated with reduced liver fat content (Barzilai *et al.* 1999).

The correlation between pro-inflammatory levels and visceral adiposity has seen these central adipose depots come under intense scrutiny in terms of deciphering the underlying cause of adipose inflammation. As mentioned above, adipocyte hypertrophy appears a crucial step in initiating the immune response. It has been suggested that adipocyte size is the crucial factor in determining the fate of the adipocyte (Weisberg *et al.* 2003). The smaller adipocytes seen in visceral adipose compared to S.C adipose is believed to render them more susceptible to hypertrophy (Murano *et al.* 2008). Indeed, comparison of visceral and S.C. adipose depots in obese rodents has demonstrated that visceral depots are more prevalent sites of adipocyte death. Supporting the greater adipocyte hypertrophy in visceral adipose, a recent paper showed preferential macrophage infiltration in visceral versus S.C. adipose in obese humans (Harman-Boehm *et al.* 2007). Furthermore, in a study

investigating CLS in visceral and S.C. adipose depots of obese rodents, CLS density was shown to be greater in visceral adipose (Strissel *et al.* 2007).

1.1.4 Mechanisms of inflammation-induced insulin resistance

In order to understand how inflammation induces insulin resistance in both liver and adipose tissue, numerous studies have focused on intracellular signalling pathways that may be disrupted. Under normal conditions (Figure 1.1a), insulin acts through the membrane-bound insulin receptor (IR). Upon ligation, the transmembrane subunits of the IR are auto-phosphorylated on three tyrosine residues (Tyr-1158, Tyr-1162, and Tyr-1163). The activated IR functions as a kinase to recruit insulin receptor substrate-1 (IRS-1) through its Src homology 2 (SH2) domain, resulting in the phosphorylation of tyrosine residues on IRS-1. This enables activation of downstream pathways, including the phosphoinositide-3 kinase (PI3K) pathway, which leads to the activation of the serine/threonine kinase AKT2. In adipose tissue, this signalling cascade leads to the recruitment of the glucose transporter 4 (GLUT4) to the plasma membrane of muscle cells and adipocytes, resulting in the enhanced glucose uptake form the blood. In the liver, AKT2 phosphorylates the forkhead box O1 (FOXO1) transcription factor, preventing its nuclear translocation. This inhibits gluconeogenic gene expression, therefore suppressing hepatic glucose production (White 1998).

Several serine/threonine kinases are activated by inflammatory stimuli (Figure 1.1b), such as TNFα binding to its cognate receptor or the binding of saturated fatty acids to TLR4 (Aguirre *et al.* 2000; Gao *et al.* 2002; Hirosumi *et al.* 2002; Draznin 2006). The two main kinases implicated in contributing to insulin resistance are the c-Jun N-terminal kinase (JNK) and the inhibitor of nuclear factor κ-B kinase subunit β (IKKβ). JNK belongs to the MAPK family and regulates the pro-inflammatory transcription factor activator protein-1 (AP-1). Activation of JNK normally occurs upon exposure to pro-inflammatory cytokines such as TNFα. However, saturated

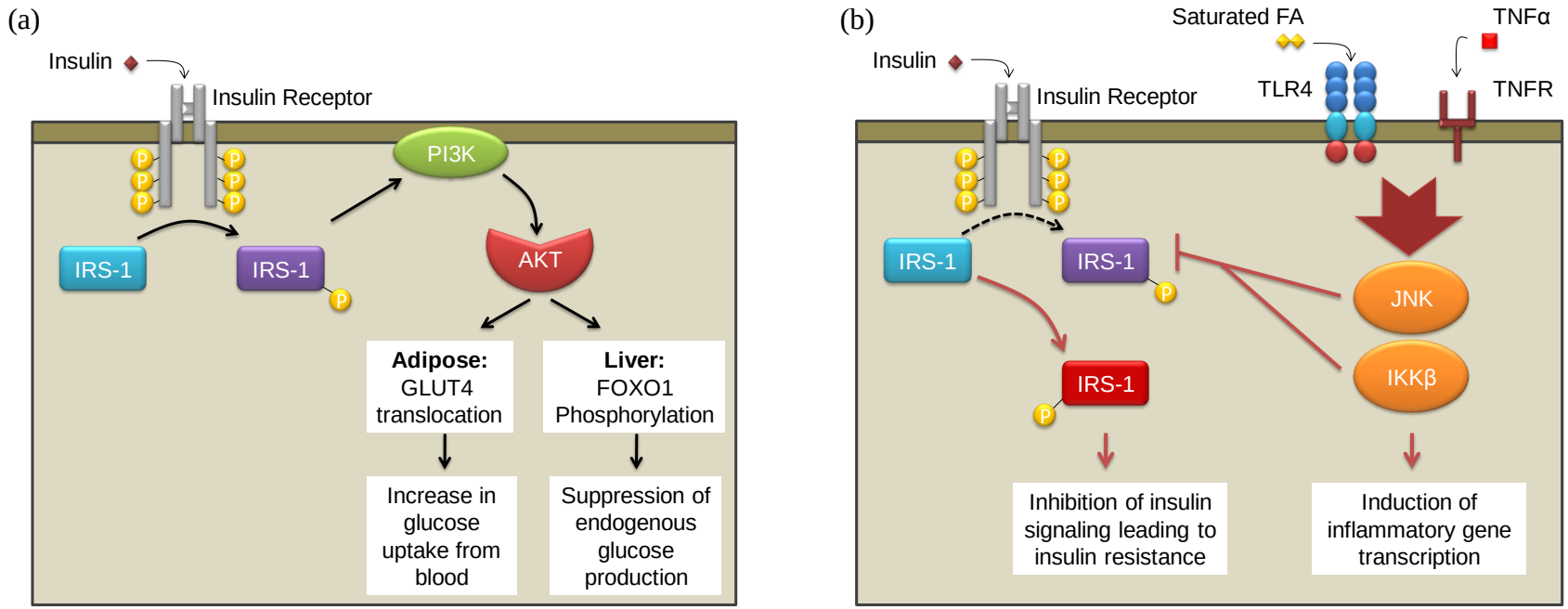

Figure 1.1 Proposed model of insulin signalling. Under normal physiological condition (a), insulin binds to the insulin receptor (IR), autophosphorylating it. This recruits insulin receptor substrate-1 (IRS-1), which is in turn phosphorylated on a tyrosine residue. This results in activation of downstream signalling pathways with different endpoints dependent upon the target tissue. In an inflammatory activated insulin target cell (b), saturated fatty acids (FA) and/or pro-inflammatory cytokines such as tumor necrosis factor-α (TNFα) act via toll-like receptor-4 (TLR4) and TNFα-receptor (TNFR) respectively to activate intracellular inflammatory kinases. Activation of c-Jun N-terminal kinase (JNK) and inhibitor of NF-κB kinase β (IKKβ) results in the inhibitory phosphorylation of IRS-1 on a serine residue, blocking normal tyrosine phosphorylation and thus blocking insulin signalling.

fatty acids have also been shown activate JNK (Aguirre *et al.* 2000; Hirosumi *et al.* 2002). In obesity, JNK activity is increased in both liver and adipose tissue, most likely as a result of the combined increase in pro-inflammatory cytokines and saturated fatty acids. This in turn leads to the inhibitory phosphorylation of IRS-1 on a serine residue (Ser-307), which prevents normal tyrosine phosphorylation, thus preventing normal insulin signalling. The importance of this pathway is demonstrated by models where loss of JNK1, one of the two isoforms of JNK, prevents the development of insulin resistance in rodent models of obesity (Hirosumi *et al.* 2002). Similar to JNK, IKKβ also phosphorylates Ser-307 on IRS-1, inhibiting normal insulin signalling (Gao *et al.* 2002). The IKKβ pathway, which is activated by TNFα, plays a key role in inflammation through activation of the pro-inflammatory transcription factor nuclear factor-κB, (NF-κB). Targeted disruption of IKKβ has been shown to improve insulin sensitivity, whilst overexpression in cultured cells attenuates insulin signalling (Yuan *et al.* 2001). Furthermore, liver-specific deletion of IKKβ resulted in improved hepatic insulin sensitivity when fed a high-fat diet (Arkan *et al.* 2005).

1.1.5 Anti-inflammatory salicylates

Although significant work has been carried out in the past number of years to establish the relationship between insulin resistance and inflammation, the idea itself is not a new concept. Indeed, initial clues date back more than century ago, when it was discovered that high doses of sodium salicylate, an anti-inflammatory drug related to aspirin, were capable of diminishing glycosuria in diabetic patients (Williamson 1901). This effect was rediscovered in 1957 when an insulin-treated diabetic patient was given high dose aspirin to treat arthritis (Reid *et al.* 1957). The result was that the patient no longer required daily insulin injections. Despite this, it was not until recently that the mechanisms behind the hypoglycemic effects of salicylates were reinvestigated.

Both sodium salicylate and aspirin are non-steroidal anti-inflammatory drugs widely used to treat conditions including rheumatic pain, yet their anti-inflammatory mechanisms are distinct. Whilst aspirin was initially shown to suppress prostaglandin production through inhibition of the cyclooxygenase enzymes (COX-1 and 2), non-acetylated salicylates do not inhibit these enzymes. However, at high concentrations, both forms inhibit the IKKβ/NF-κB axis. It is the effects on this pathway that led to the investigation of salicylates in treating inflammation-induced insulin resistance. Unlike the acetylated aspirin, which increases the risk of bleeding, the non-acetylated salicylate does not inhibit platelet aggregation, reducing such risk. Initial studies in genetically obese rodents found that salicylate treatment resulted in a reduction in both fasting blood glucose levels and insulin concentration (Yuan *et al.* 2001). Further work also demonstrated that lipid-induced insulin resistance in rodents was also attenuated with salicylate treatment (Kim *et al.* 2001).

The promise in these studies led to the effects of salicylates being investigated in humans. Initial studies with aspirin found that, in healthy lean men, lipid-induced insulin resistance was improved with aspirin pre-treatment, although no beneficial effect was observed in the control group (without lipid infusion) (Mohlig *et al.* 2006). Furthermore, in diabetic patients, a two week course of high dose aspirin improved insulin sensitivity (Hundal *et al.* 2002). However, such high doses over an extended period of time have potentially grave side effects. Whilst salicylates are not associated with increased spontaneous bleeding risk, they do induce gastric irritation when administered at high dose. To overcome this issue, trials have switched to the more tolerable form of salicylate, namely salsalate, a drug composing of two salicylate molecules bound by an ester bond. Due to its insolubility in gastric acid, it is not absorbed in the stomach, therefore putatively causing less irritation. However, the alkaline conditions present within the intestine break the ester bond, freeing the two salicylate molecules and allowing them to be absorbed. In obese, non-diabetic patients, treatment with salsalate had no effect on body weight, reduced glucose levels, but increased insulin levels (Koska *et al.* 2009). In this study, the glucose-lowering effects of salsalate were determined to be a result of increased systemic

insulin concentrations. A similar study in obese young adults found that one month salsalate treatment reduced glucose and NEFA levels, with no changes in insulin (Fleischman *et al.* 2008). However, this group did find reduced insulin clearance following treatment, which may account for the improved glucose disposal. Another study, this time investigating the effects in diabetic patients, also found beneficial effects of salsalate treatment in reducing glucose levels (Goldfine *et al.* 2008). Whilst these studies identified a potential role for salsalate as a treatment option, their conclusions were limited by the small numbers of patients in each study, as well as the short duration of drug administration. To address this, a large randomised trial, Targeting Inflammation using Salsalate for Type 2 Diabetes (TINSAL-T2D), began in 2008 (Goldfine *et al.* 2010). The recently published findings from the initial part of this study found that a 14 week administration of salsalate at several doses (3.0, 3.5, 4.0g per day) were well tolerated in patients with type 2 diabetes, and improved measures of glycaemic control, as well as increasing circulating adiponectin levels.

The results of the TINSAL-T2D trial clearly support targeting inflammation within the setting of metabolic syndrome as a therapeutic option. However, the molecular mechanisms underlying the glucose lowering actions remain undefined. Given the ability of salicylate to inhibit NF-κB action via IKKβ inhibition, and the role that both these molecules play in altering insulin signalling, this inflammatory pathway has been identified as a potential molecular target of salicylate. Indeed, insulin resistance induced in mice through overexpression of hepatic IKKβ was attenuated by salicylate treatment (Cai *et al.* 2005). Despite this, studies in humans have suggested the beneficial effects are a result of increased insulin concentrations (Fernandez-Real *et al.* 2008). As a result, the mechanisms of salicylate-induced insulin sensitisation remain to be fully elucidated. In this thesis, a role for modulation of glucocorticoid action will be explored.

1.2 Glucocorticoids

Glucocorticoids are steroid hormones that function as regulators of metabolic and immune status. Like other steroids, they are based on the cyclopentanoperhydrophenanthrene structure (Figure 1.2), consisting of three cyclohexane rings and a single cyclopentane ring. The unique properties of each steroid are achieved through substitution of chemical groups at various positions.

1.2.1 Biosynthesis of glucocorticoids

Glucocorticoids are derived from the precursor cholesterol within the zona fasciculate of the adrenal cortex. This synthetic pathway is catalysed by members of the cytochrome P450 oxidative enzyme family, as well as 3β-hydroxysteroid dehydrogenase (Figure 1.3). In humans, the active glucocorticoid is cortisol, whilst in rodents, the absence of 17α-hydroxylase results in corticosterone being the active glucocorticoid (Chung *et al.* 1987; Luu-The *et al.* 2005).

1.2.2 Regulation of glucocorticoids

Endogenous levels of circulating glucocorticoids are controlled by the hypothalamic-pituitary-adrenal (HPA) axis. The synthesis of glucocorticoids in the adrenal glands is regulated by adrenocorticotrophic hormone (ACTH), which is secreted from the anterior pituitary. Within these corticotropes, ACTH is cleaved from the pre-cursor pro-opiomelanocortin (POMC), under the control of corticotrophin releasing hormone (CRH). CRH, secreted from within the paraventricular nucleus of the hypothalamus, is produced in response to various physical and psychological stressors. Such stressors include factors released by the immune system if the body is fighting infection, including pro-inflammatory cytokines. The HPA axis itself is regulated through several negative feedback systems. Elevated circulating levels of glucocorticoids act upon the pituitary to inhibit transcription of POMC, as well as preventing cleavage of POMC, thus preventing production of ACTH. Furthermore, the increased levels of glucocorticoids act upon two main regions of the brain, namely the hypothalamus, inhibiting transcription of CRH, as well as the

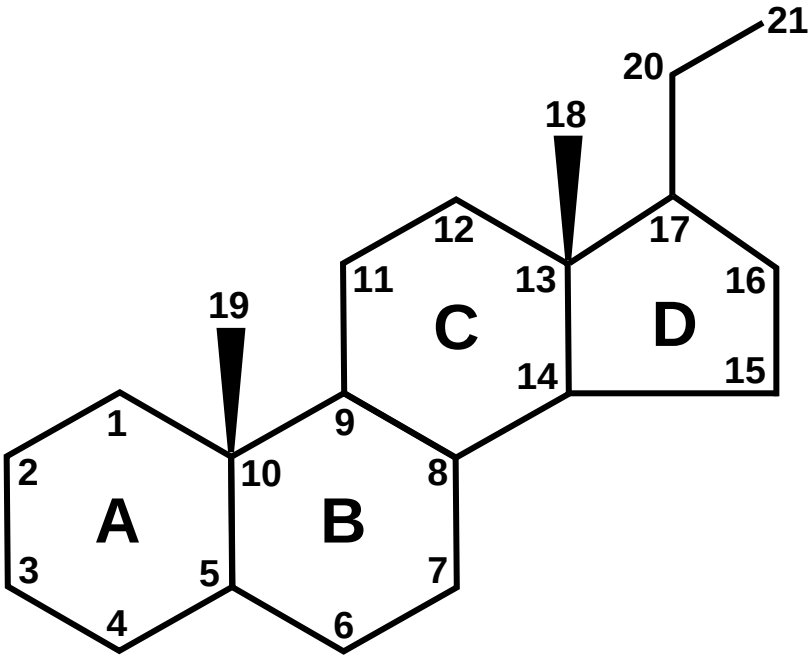

Figure 1.2 Glucocorticoid cyclopentanoperhydrophenanthrene structure. The three cyclohexane rings and the single cyclopentane ring are identified by letters and the individual carbon atoms by numbers.

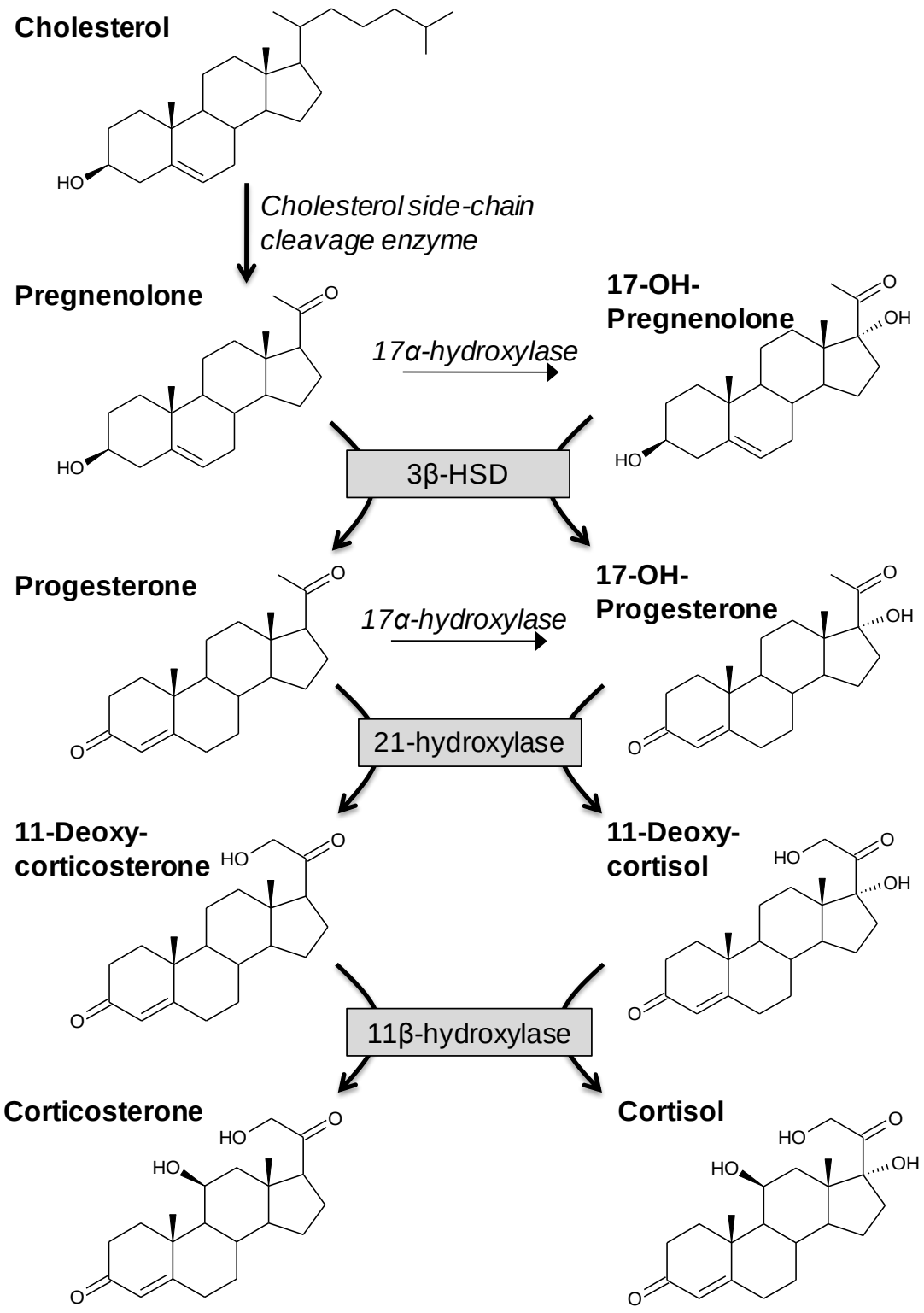

Figure 1.3 Biosynthesis of glucocorticoids. Pathways involved in production of glucocorticoids in the adrenal cortex. HSD = hydroxysteroid dehydrogenase.

hippocampus, reducing stimulation of CRH release from the hypothalamus (Stewart 2002). A further level of glucocorticoid regulation is evident in the 24 hour circadian rhythm of circulating glucocorticoid levels. In humans, glucocorticoid levels are highest in the morning (Lupien *et al.* 1998). In nocturnal animals, such as rodents, glucocorticoid levels are lowest in the morning, rising during the day to reach a peak in the evening (Windle *et al.* 1998).

1.2.3 Glucocorticoid activation of glucocorticoid receptor

The actions of glucocorticoids are mainly mediated through interactions with two nuclear hormone receptors, namely the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR) (Lu *et al.* 2006). Alternative splicing of the pre-mRNA generates multiple isoforms of the GR, with GRα, the most functional isoform, expressed throughout the body. GRβ does not bind glucocorticoids and so has been less extensively researched. However, its ability to bind to DNA means that it may act in a dominant-negative inhibitor of glucocorticoid action by interfering with GRα binding to DNA. (Given that glucocorticoids have been shown to exert effects only via the GRα isoform, mention of GR from this point on refers to GRα). Whilst the physiological and pharmacological actions are primarily mediated via the GR, glucocorticoid binding and activation of other receptors exerts other effects. MR is part of the same superfamily of nuclear receptors as GR, with a high affinity for glucocorticoids. Yet glucocorticoid action via MR is often prevented by inactivation of the glucocorticoid by specific enzymes, discussed in detail later. Other nuclear receptors that glucocorticoids bind with include the steroid and xenobiotic receptor (SXR), which also responds to a wide range of endogenous steroids (Blumberg *et al.* 1998). SXR was initially established as a sensor that regulated xenobiotic clearance within the liver and intestine, yet over the past few years, research has revealed a role for SXR in inflammation, as well as lipid and energy homeostasis (Zhou *et al.* 2009).

Like other steroid receptors, the modular structure of GR contains three major domains (Figure 1.4), namely the N-terminal domain, the central DNA-binding domain (DBD) and the C-terminal, ligand-binding domain (LBD) (Beck *et al.* 2009). The N-terminal domain represents the most variable region within the family of

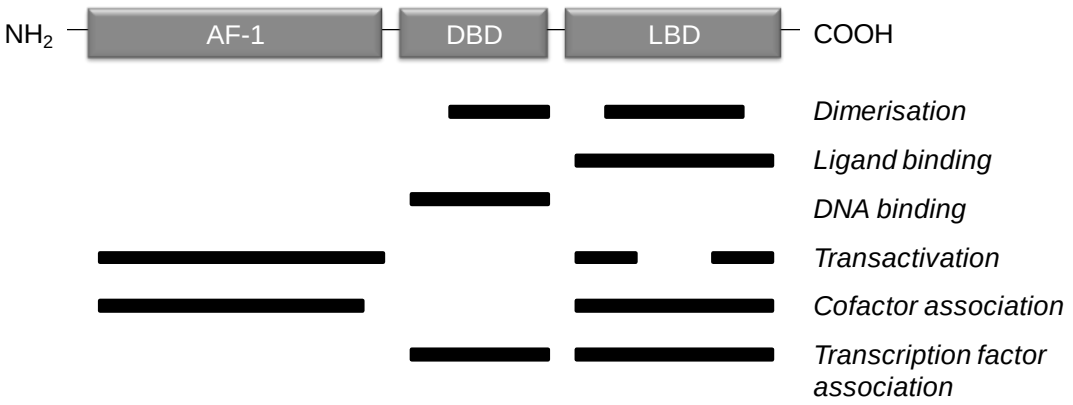

Figure 1.4 Structure of glucocorticoid receptor. The three domain structure of glucocorticoid receptor is shown, namely the N-terminal activation function 1 (AF-1), the DNA-binding domain (DBD) and the ligand-binding domain (LBD). The functions of the various domains are also highlighted.

nuclear receptors, and contains a transactivation domain, activation function-1 (AF-1). The importance of this AF-1 region in activation has been well established, with much of the GR's transcriptional activity dependent upon this region. AF-1 interacts with several transcription factors, including TATA-binding protein (TBP) and CREB-binding protein (CBP) (Kumar and Thompson 2005). The N-terminal domain also serves as a site of coregulatory protein binding. The DBD is highly conserved in terms of amino acid sequence, and serves two main functions; firstly it confers receptor recognition for specific DNA sequences within the promoter region of glucocorticoid-regulated genes. Secondly, the DBD has two zinc finger regions which are crucial for GR dimerisation. Furthermore, the DBD interacts with other proteins, including the c-Jun subunit of the AP-1 complex. The LBD consists of 11 helices that fold into a globular structure that forms the ligand-binding pocket (Bledsoe *et al.* 2002). This hydrophobic ligand-binding site establishes a unique hydrogen bond network between the receptor and the bound ligand, ensuring specificity between the two. However, the ligand itself also has an important role to play in determining the downstream consequences of GR activation. The ligand binding pocket contains a branched side pocket critical for high affinity binding of ligands. The structure of the ligand is important as it determines the conformation that the receptor assumes after ligand binding. Such changes in conformation result in changes to the receptor surface, including DNA-binding surface, the dimerisation surface and also the ligand-binding surface. The LBD also contains a second transactivation domain (AF-2) located within helix 11. This region binds a number of coregulatory proteins (discussed later), which are often essential for transcriptional activation.

In the resting state, the GR molecule is held in the cytoplasm as part of a large multi-protein chaperone complex consisting primarily of heat-shock proteins (Hsp) (Pratt *et al.* 2006). These include Hsp90 and Hsp70, as well as several Hsp90-binding proteins that serve to stabilise the complex, including p23, Hop, FKBP51, FKBP52 and CyP-40. This complex binds to the LBD domain, inducing a conformational state favourable to high-affinity ligand binding. Given their small hydrophobic

nature, glucocorticoids readily diffuse across the membrane of their cellular targets, although they may also be actively transported into the cell (Fant *et al.* 1983). Upon binding to the cytosolic GR, the chaperone complex dissociates, inducing a conformational change which unmasks the nuclear localisation signal. Two nuclear localisation sites have been identified within the GR (Smoak and Cidlowski 2004). The ligand-independent NL1 is located with the DBD, whilst the ligand-dependent NL2 is located in the LBD. The activation of these nuclear localisation sites facilitates translocation to the nucleus of the cell, where importin-α and importin-β are involved in the nuclear import of GR (Freedman and Yamamoto 2004). The export of GR from the nucleus is believed to involve calreticulin- and exportin-1-based mechanisms. However, the GR itself may determine its maintenance within the nucleus through the nuclear retention signal.

1.2.4 Glucocorticoid receptor-mediated effects on transcription

Following glucocorticoid binding and nuclear translocation, the activated GR acts through several mechanisms to modulate transcriptional activity in a positive (transactivation) or negative (transrepression) manner (Figure 1.5) (De Bosscher *et al.* 2003; Newton and Holden 2007; Beck *et al.* 2009; De Bosscher and Haegeman 2009).

1.2.4.1 Transactivation

Transactivation is primarily mediated by the binding of GR homodimers to glucocorticoid response elements (GREs) within the promoter region of target genes (Figure 1.5a). These consensus GREs contain imperfect palindromic sequences (5' GGT ACA nnn TGT TCT 3') consisting of two hexameric half-sites separated by three base pairs of any nucleotide (n) (Newton and Holden 2007; Beck *et al.* 2009). Two GR monomers bind to the DNA, forming a GR homodimer. This mechanism

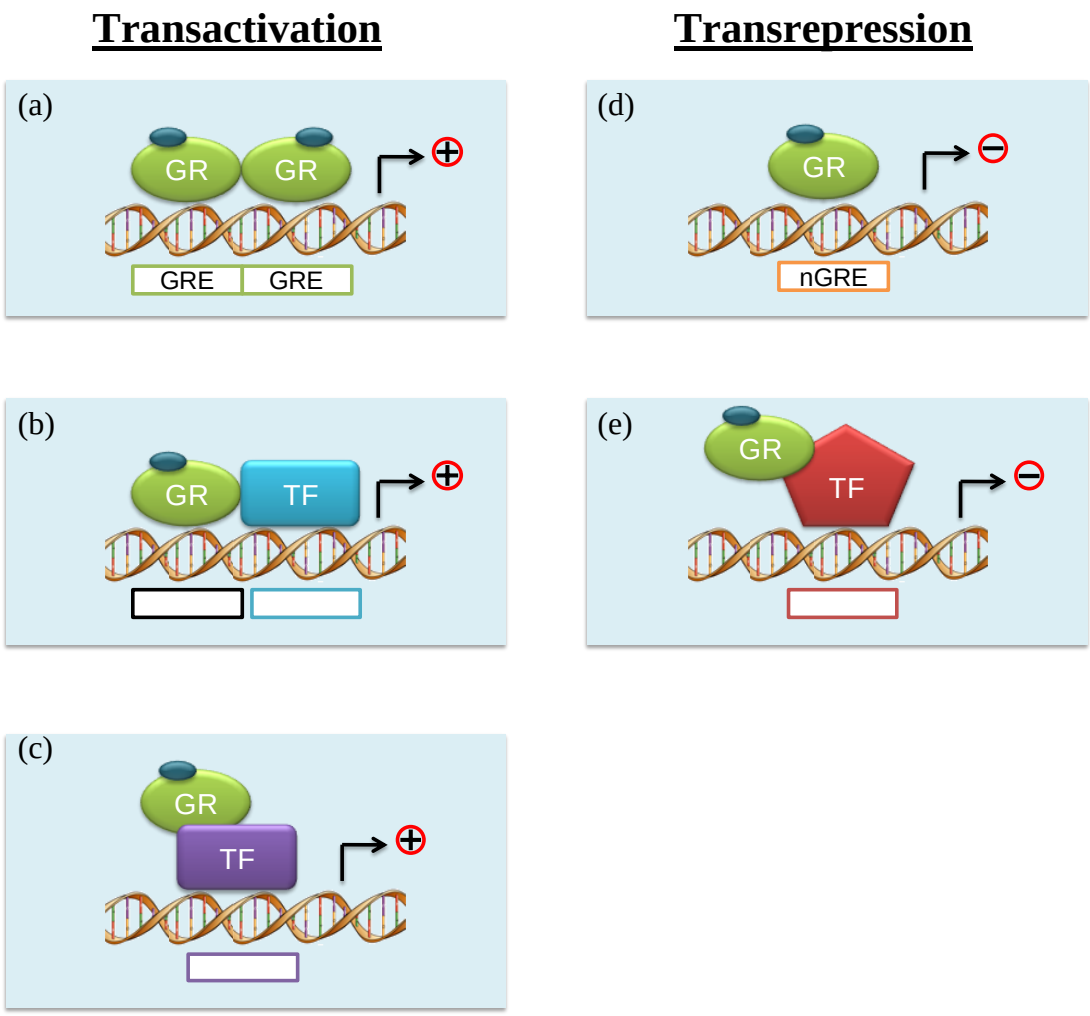

Figure 1.5 Nuclear actions of glucocorticoid receptor (GR). Mechanisms of positive transcriptional activation (Transactivation, a-c) and negative transcriptional activation (Transrepression, d-e). GR can dimerise and bind to glucocorticoid response elements (GRE) within promoter of target gene, inducing transcription (a). GR monomer can bind to GRE half-sites in the DNA sequence that are located beside transcription factor (TF) binding sites, acting synergistically to induce gene transcription (b). Alternatively, GR monomers can bind directly to TFs, without binding to DNA, inducing (c) or inhibiting (e) gene transcription. Direct repression of gene transcription also occurs through GR monomer binding to negative GREs (nGRE), which blocks transcription (d).

explains transactivation of simple GREs, which contain consensus GRE sites, such as is the case for the mouse mammary tumour virus (MMTV) promoter, the tyrosine aminotransferase (TAT) promoter and the glucose-6-phosphatase promoter (De Bosscher *et al.* 2003; Beck *et al.* 2009). Whilst this has classically been viewed as the mechanism behind GR-mediated activation of target genes, it is now known that numerous other genes are upregulated through alternative mechanisms. In particular, recent work has highlighted that dimerisation of GR is not essential, utilising dimerisation-deficient GR to demonstrate an equal ability to transactivate gene expression of phenyl-N-methyl-transferase (PMNT) as wild-type GR (Adams *et al.* 2003). Although dimerisation is not essential, DNA-GR interactions, either directly or indirectly, are crucial. In these situations, GR monomers bind to single or multiple GRE half-sites, often in combination with transcription factors, which act in a collaborative manner to enhance gene activation (Figure 1.5b) (De Bosscher *et al.* 2003).

A further transactivation mechanism does not involve direct DNA-binding, but rather 'tethering' of the GR monomer to DNA-bound transcription factors. An example of this is GR monomer binding to signal transducer and activator of transcription 3 (STAT3), enhancing its transcriptional activity and increasing gene expression of IL-10 (Figure 1.5c) (Beck *et al.* 2009).

1.2.4.2 Transrepression

Similar to transactivation, GR-mediated transrepression is regulated through both direct and indirect DNA-binding mechanisms. Binding of GR monomers to negative GREs mediates the direct DNA binding mechanism, for example in glucocorticoid-mediated repression of osteocalcin (Figure 1.5d) (Meyer *et al.* 1997). However, only a handful of genes have been shown to contain negative GREs. In terms of inflammatory gene promoters, there is a distinct absence of consensus sequences to which GR can bind. Instead, these promoters contain binding sites for the pro-inflammatory transcription factors NF-κB and AP-1. The proposed predominant

mechanism of transrepression involves tethering of GR monomers to transcription factors, inhibiting their transcriptional activity (Beck *et al.* 2009). Indeed, this mechanism has been shown to be important for repressing pro-inflammatory gene transcription through tethering of GR to NFκB and AP-1 (Figure 1.5e). However, whilst DNA-binding is not required in this mechanism, the DBD of the GR still appears to be crucial for repression of some inflammatory pathways. This was demonstrated in studies utilising a GR with a mutation within the second zinc finger of the DBD, when repression of AP-1 transcription was observed, but repression of NFκB was prevented (Bladh *et al.* 2005).

1.2.4.3 Role of coregulators

The ability of GR to act as a transcriptional activator or repressor depends not only upon the presence of specific transcription factors, but also the presence of coregulators. The role of these coregulators has only emerged in recent years, and remains an area under extensive investigation. These molecules act as signalling proteins between the DNA-bound GR and local transcription machinery, reorganising the chromatin environment upon recruitment by the GR (De Bosscher and Haegeman 2009; Barnes 2010). Coregulator molecules are designated as coactivators or corepressors based on their ability to facilitate or repress transcription. Once recruited, coactivators such as transcription intermediary factor-2 (TIF-2), steroid receptor coactivator-1 (SRC-1) and p300 enhance transcriptional activity through several mechanisms, including recruitment of basal transcription machinery (Coghlan *et al.* 2003; De Bosscher and Haegeman 2009). Furthermore, coactivators have been shown to possess histone acetyl transferase (HAT) activity, reorganising the chromatin structure in such a way that recruited transcriptional machinery has greater access to DNA (Barnes 2010). Corepressors, such as nuclear receptor corepressor (NCoR), and silencing mediator of retinoic acid and thyroid hormone receptor (SMRT), negatively regulate gene expression. Primarily, corepressors are bound to unliganded receptors, preventing gene expression in the absence of the cognate ligand. However, corepressors also bind to receptors in the presence of their respective antagonists, actively repressing gene expression. These

corepressors have histone deacetylase (HDAC) activity, reorganising the chromatin into a restrictive structure that prevents access of transcriptional machinery (De Bosscher and Haegeman 2009; Barnes 2010). One such example of the importance of these coregulators proteins was recently shown in muscle cells. Treatment of these cells with dexamethasone, a more potent, synthetic analogue of cortisol, induces cellular proteolysis. This is attenuated by treatment with the GR antagonist RU486, demonstrating the effects are GR-mediated (Yang *et al.* 2007). However, the effects are also attenuated upon gene silencing of the coregulatory protein p300. Furthermore, in cells transfected with a mutant form of p300 lacking HAT activity, dexamethasone-mediated proteolysis was suppressed (Yang *et al.* 2007).

1.2.4.4 Non-genomic effects of glucocorticoids

The genomic actions of glucocorticoids are responsible for transactivation and transrepression of target genes. However, there are also multiple, rapid non-genomic effects exerted by glucocorticoids that are believed to occur in major tissues throughout the body. Firstly, glucocorticoids affect membrane fluidity through direct interactions with lipids in both plasma and mitochondrial membranes. High concentrations of glucocorticoids have been shown to incorporate into membranes, where they alter activities of membrane-associated proteins, including the Ca^{2+}, K^{+} and Na^{+} channels (Stahn *et al.* 2007). These changes could contribute to the anti-inflammatory effects of glucocorticoids by inhibiting ATP production (Song and Buttgereit 2006). Secondly, glucocorticoids have been shown to associate with membrane-bound receptors, distinct from classical glucocorticoid receptors. These include gamma-aminobutyric acid (GABA) receptors (Majewska 1987) and low-affinity glucocorticoid receptors (LAGs) (Roszak *et al.* 1990), as well as membrane-bound GR (mGR) (Bartholome *et al.* 2004), a variant of the classical cytosolic GR. Glucocorticoids also have rapid effects on caveolin, a scaffolding protein implicated in the organisation of signalling molecules. Glucocorticoids have been shown to alter phosphorylation levels of caveolin, resulting in activation of protein kinase B (PKB)/Akt pathways, in a rapid, non-genomic manner (Matthews *et al.* 2008). A final class of non-genomic effects involves binding of cytosolic GR. This ligation

releases proteins, including Src, which in turn interacts with lipid membranes, blocking arachidonic acid production (Croxtall *et al.* 2000). This molecule is a precursor for prostaglandins and leukotrienes, which contribute to the inflammatory response. As such, inhibition of arachidonic acid production represents a further non-genomic, anti-inflammatory mechanism of glucocorticoids (Alangari 2010).

1.2.5 Physiological effects of glucocorticoids

Glucocorticoids exert a broad range of effects throughout the body. They act upon multiple target systems to increase energy substrate availability, as well as to modulate the immune response, allowing for adaptation to the changing environment.

1.2.5.1 Lipid, carbohydrate and protein metabolism

In an environmental situation of starvation or perceived danger, glucocorticoids act to provide energy substrates for the body in the form of glucose and NEFAs, thus opposing the effects of insulin, which acts to lower blood glucose and store fuel (Andrews and Walker 1999). The molecular mechanisms behind these effects are believed to mainly involve transactivation of target genes. In the liver, glucocorticoids activate gluconeogenic pathways through increased transcription of enzymes involved in this pathway such as phosphoenolpyruvate carboxykinase (PEPCK) (Sugiyama *et al.* 1998), glucose-6-phosphatase (G6Pase) (van Schaftingen and Gerin 2002) and tyrosine aminotransferase (TAT) (Dostert and Heinzel 2004). Furthermore, they simultaneously enhance glycogen synthase and glycogenolysis, described as ‘futile’ cycling, serving to prime the body for more effective release of fuel during times of stress or starvation (Macfarlane *et al.* 2008). In peripheral tissues, including skeletal muscle and adipose, glucose uptake is reduced, whilst breakdown of energy substrates is increased (Buren *et al.* 2002). In skeletal muscle, proteolysis is induced to release amino acids into the circulation, providing a substrate for gluconeogenesis (Jackman and Kandarian 2004). In adipose tissue, the effects of glucocorticoids on fatty acid metabolism are dependent upon the presence

of other hormones, namely insulin and catecholamines. In times of stress and starvation, when insulin is low and catecholamines elevated, glucocorticoids promote lipolysis, producing NEFAs which serve as a fuel source for peripheral tissues. Conversely, in an environment of high insulin and lower catecholamines in which lipolysis is suppressed, glucocorticoids promote lipid storage (Coppack *et al.* 1994; Macfarlane *et al.* 2008).

1.2.5.2 Immunomodulatory effects

Whilst commonly perceived as anti-inflammatory, physiological levels of glucocorticoids are more accurately immunomodulatory in action. Glucocorticoids act to suppress the production of pro-inflammatory cytokines, including TNFα, IL-6 and IL-1β, inhibiting the inflammatory response (Barnes 1998). However, glucocorticoids also modulate the recruitment of inflammatory cells to sites of inflammation (McEwen *et al.* 1997; Tuckermann *et al.* 2007). This occurs primarily through suppressing the release of chemokines, including MCP-1, IL-8 and macrophage-inflammatory protein-1α (MIP-1α), but also suppressing the expression of adhesion molecules involved in lymphocyte extravasation, including intracellular adhesion molecule-1 (ICAM-1) and E-selectin (Tuckermann *et al.* 2007). These suppressive effects are mediated through both transrepressive mechanisms, whereby GR binds to the pro-inflammatory transcription factors NF-κB and AP-1, preventing transcription, and transactivation mechanism, namely an increase in the levels of MKP-1, which inactivates intracellular inflammatory signalling pathways, and IκBα, which retains NF-κB in an inactive form in the cytoplasm (De Bosscher *et al.* 2003; Beck *et al.* 2009). Aside from inhibiting production of pro-inflammatory molecules, glucocorticoids also promote the resolution of inflammation. This is achieved through the induction of anti-inflammatory gene expression, namely IL-1 receptor antagonist, IL-4, IL-10 and annexin-1 (Beck *et al.* 2009), and also on a cellular level through induction of leukocyte apoptosis and enhanced phagocytic clearance of such apoptotic cells (Liu *et al.* 1999).

1.2.5.3 Blood pressure

Glucocorticoids act upon the both the kidney and vasculature through multiple mechanisms to regulate blood pressure. They induce nitric oxide-mediated vasodilation of afferent and efferent arterioles in the kidney, as well as increasing salt and water retention (De Matteo and May 1997; Whitworth *et al.* 2000). These effects are mediated through both GR and MR. Whilst MR has a 10-fold higher affinity for cortisol and corticosterone than GR (Nishi and Kawata 2007), and MR is enriched within the kidney, activation of this receptor within the kidney is protected by the enzyme 11β-hydroxysteroid dehydrogenase type 2 (11βHSD2 described 1.2.7.1). This enzyme inactivates glucocorticoids, thus preventing activation of MR (Roland *et al.* 1995). In conditions of 11βHSD2-deficency, glucocorticoids induce hypertension due to inappropriate activation of MR. In vascular smooth muscle, glucocorticoids increase vascular tone primarily through GR-mediated mechanisms (Walker and Williams 1992). Levels of the calcium-activated potassium channel, which acts to allow relaxation of vascular smooth muscle cells through efflux of potassium, is reduced by glucocorticoids, resulting in vasoconstriction of vessels (Brem 2001). Glucocorticoids also activate the renin-angiotensinogen system through upregulation of both angiotensinogen and angiotensin-converting enzyme, which also results in vasoconstriction (Sato *et al.* 1994). Furthermore, glucocorticoids reduce the levels of several vasodilator proteins, including nitric oxide through suppression of endothelial and inducible nitric oxide synthase (Mitchell and Webb 2002), and prostaglandin I2 (Falardeau and Martineau 1989).

1.2.5.4 Bone remodelling

Glucocorticoids suppress the proliferation of osteoblasts through transcriptional inhibition of osteocalcin, a key protein in bone formation, via glucocorticoid action on the negative GRE within the promoter of this gene (Meyer *et al.* 1997). Apoptosis of both osteoblasts and osteocytes is also induced by glucocorticoids, reducing the number of bone cells, whilst the differentiation of osteoclasts is increased, enhancing bone resorption (Schacke *et al.* 2002).

1.2.6 Pharmacological effects of glucocorticoids

The anti-inflammatory properties of glucocorticoids were first reported over half a century ago (Hench *et al.* 1949), leading to extensive research into their therapeutic benefits. In this time, the pharmaceutical industry has developed a number of synthetic glucocorticoids, including dexamethasone, betamethasone, triamcinolone, prednisone, prednisolone and methylprednisolone. This has led to glucocorticoids becoming one of the most commonly prescribed agents for the treatment of inflammatory and immune diseases (Saklatvala 2002). Indeed, glucocorticoids are very effective in the treatment of inflammatory conditions such as rheumatoid arthritis and inflammatory bowel disease. However, the pharmacological doses of glucocorticoids often required to control inflammation in patients often result in the development of detrimental side effects. These include osteoporosis, growth retardation, muscle wasting and mood disorders (Saklatvala 2002; Schacke *et al.* 2002). Furthermore, perhaps the most serious side effect resulting from excess glucocorticoids is the development of diabetes and cardiovascular disease. Indeed, there is considerable evidence for the development of these conditions after glucocorticoid therapy. Transplant patients are often treated with glucocorticoids due to their immunosuppressive properties, and this is believed to be responsible for the development of post-transplant diabetes mellitus (PTDM) (Schacke *et al.* 2002). Treatment is associated with the risk of hyperglycemia in patients without pre-existing diabetes mellitus, and worsened glycemic control of diabetic patients. Recent population-based studies found that the use of glucocorticoids was associated with an increased risk of cardiovascular events (Wei *et al.* 2004). In particular, high dose glucocorticoid treatment resulted in patients being 2.5 times more likely to have a cardiac event. These side effects are thought to be mainly a result of glucocorticoid-induced transactivation of metabolic genes, including key enzymes in the gluconeogenic pathway.

1.2.6.1 Dissociated glucocorticoids

The demonstration that transactivation could account for the development of the metabolic side effects associated with glucocorticoid therapy, coupled with the anti-inflammatory effects being controlled by primarily by transrepression, led to intense research into the development of ligands that could distinguish between transactivation and transrespression, namely dissociated steroids and selective GR modulators (SGRMs) (De Bosscher 2010).

Initial work focussed on altering known steroid ligands in an attempt to separate out transactivation and transrepression mechanisms. Several synthetic glucocorticoids were developed that dissociated these pathways *in vitro,* namely RU24782, RU24858 and RU40066 (Vayssiere *et al.* 1997). These compounds displayed a reduced transactivation ability compared to both dexamethasone and prednisolone, whilst demonstrating impressive anti-inflammatory properties. Utilising *in vivo* models of inflammation, including croton-oil induced ear oedema, these compounds were tested and compared to prednisolone. RU40066 had no *in vivo* effects on inflammation. However, both RU24782 and, in particular, RU24858 were effective at reducing inflammation (Vayssiere *et al.* 1997). Despite this initial promise, the reduced transactivation effects shown *in vitro* were not recapitulated *in vivo* (De Bosscher 2010). The reason for the discrepancies between *in vitro* and *in vivo* work is believed to result from metabolism of these compounds within the body. However, perhaps a more telling observation is the growing evidence that distinguishing between deleterious side effects and beneficial anti-inflammatory effects is not as simple as separating transactivation from transrepression. Indeed, several anti-inflammatory molecules are induced by glucocorticoids, such as IL-10, MLP-1 and IκBα, which contribute significantly to the overall anti-inflammatory effect.

The continued development of side effects from the above steroids resulted in a focussing of attention on non-steroidal compounds. In particular, two SGRMs have shown promise, namely AL-438 and CpdA. AL-438 is a modified form of a synthetic

progestin, which was shown to reduce inflammation in an *in vivo* model of asthma, but had reduced effects on hyperglycemia compared to classic glucocorticoid ligands (Coghlan *et al.* 2003). Further work on this compound found that key differences exist in the ability of AL-438-bound GR to interact with coregulatory molecules compared to glucocorticoid-bound GR. In particular, AL-438-bound GR did not associate with the coactivator peroxisome proliferator-activated receptor-γ coactivator-1 (PGC-1), which plays a role in the ability of glucocorticoids to induce hepatic gluconeogenic gene expression. This suggests that AL-438 induces conformational changes within the GR, specifically within the LBD, that alter its ability to interact with coregulatory proteins (Coghlan *et al.* 2003).

CpdA is a plant-derived compound that was shown to display dissociated characteristics *in vitro* (De Bosscher *et al.* 2005). Further work revealed that CpdA induced a conformational change in GR that favoured monomer over dimer formation, as is seen with classic glucocorticoids (Robertson *et al.* 2010). Several *in vivo* models of inflammation, including arthritis and experimental autoimmune encephalomyelitis (EAE), have confirmed the anti-inflammatory potential of this SGRM, as well as demonstrating that beneficial effects were not accompanied by changes in metabolic parameters (Dewint *et al.* 2008; van Loo *et al.* 2010). However, whilst CpdA does not exert the same level of GR-dependent side effects as classic glucocorticoids, it does exert GR-independent effects, inducing apoptosis in several cell types, including lymphocytes and neuronal cells (Wust *et al.* 2009). This has been proposed to result from the metabolism of CpdA *in vivo* into aziridine derivatives, which have strong pro-apoptotic and neurotoxic properties. Nonetheless, these agents provide hope that manipulation of GR ligands may yet yield a safer pharmaceutical alternative to classic glucocorticoids.

Potential alternatives to glucocorticoids are produced endogenously through a series of metabolising pathways (described 1.2.7), and it may be that these compounds, in particular 5α-reduced glucocorticoids, have the potential to be dissociated steroids.

1.2.7 Metabolism of glucocorticoids

The levels of glucocorticoids in the body are kept in balance through a series of production and clearance mechanisms. The HPA axis is responsible for regulating circulating levels of glucocorticoids, with negative feedback mechanisms controlling synthesis and release of these hormones from the adrenal gland. However, the concentrations of glucocorticoids available for action are also regulated by metabolism within tissues. These steroids can be activated or inactivated locally by various mechanisms within target tissues, thus regulating access of the hormone to its receptor at a local level (Figure 1.6). The clearance of glucocorticoids is predominantly a hepatic process involving A-ring reduction by several enzymes within the A-ring reductase family, including 5α- and 5β-reductases and 3α-hydroxysteroid dehydrogenase (3αHSD) (Walker and Andrew 2006). Another major route of metabolism in numerous tissues involves 11β-hydroxysteroid dehydrogenases (11βHSDs), which, depending on the isozyme involved, generates or inactivates local glucocorticoids (Walker and Andrew 2006).

1.2.7.1 11β-Hydroxysteroid dehydrogenases (11βHSDs)

There are two isozymes of 11βHSD, type 1 and 2, which catalyse the interconversion of active glucocorticoids and their inert 11-keto derivatives. Both enzymes are microsomal membrane-bound proteins, which act to modify local exposure to glucocorticoids.

In cell homogenates, 11βHSD type 1 (11βHSD1) is bidirectional, whereas in intact cells, it functions predominantly as a reductase, regenerating active glucocorticoids from their inactive 11-keto metabolites. The determination of reductase activity is dependent upon hexose-6-phosphate dehydrogenase (H6PDH). Within intact cells, H6PDH co-localises with 11βHSD1 at the ER lumen, providing it with the reduced form of nicotine adenine disphosphonucleotide (NADPH) as a cofactor, thus driving reductase activity (Figure 1.7) (Morton 2009). Indeed, the importance of H6PDH is shown in H6PDH-deficient mice which lack 11βHSD1 reductase activity (Lavery *et al.* 2006). 11βHSD1 is expressed in a wide variety of tissues, including liver, adipose tissue, skeletal muscle, lung, brain, immune cells and vascular smooth muscle. It is

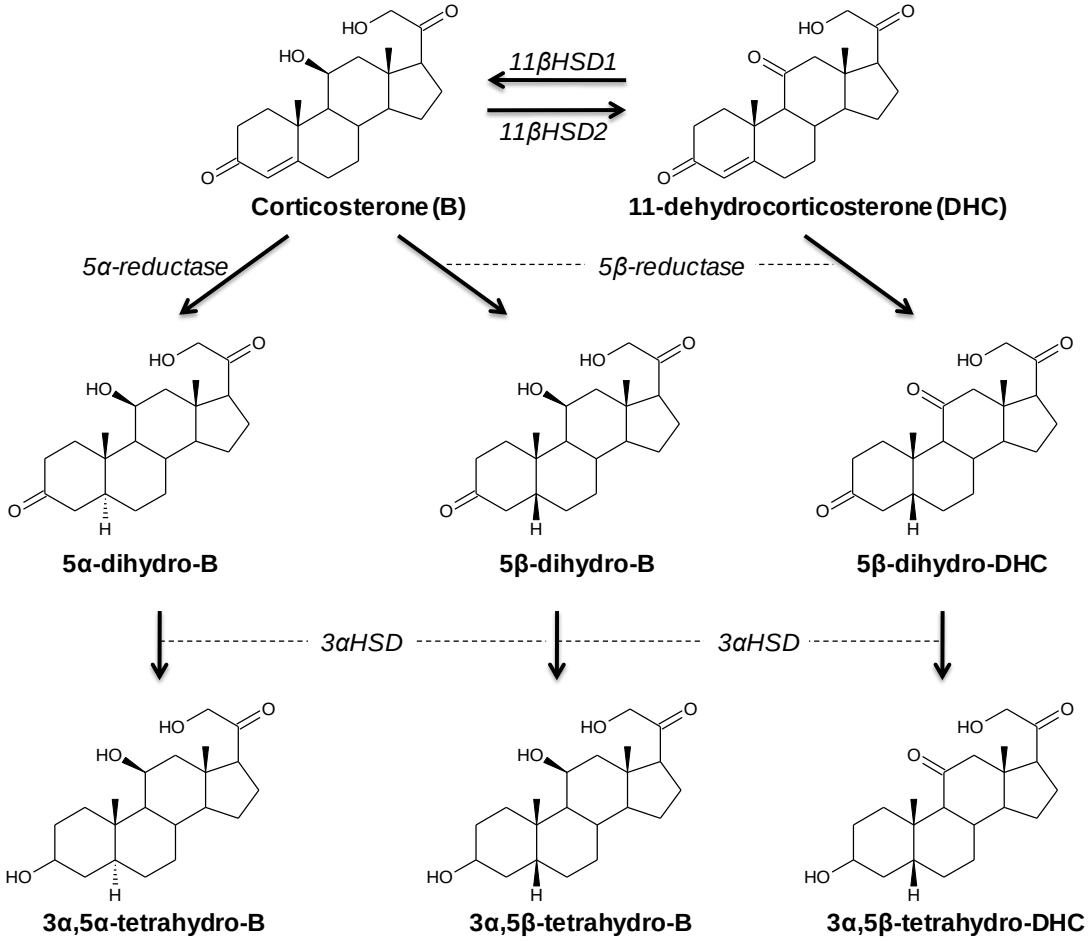

Figure 1.6 Glucocorticoid metabolism. Various pathways and enzymes involved in the metabolism of the rodent glucocorticoid corticosterone. HSD = hydroxysteroid dehydrogenase.

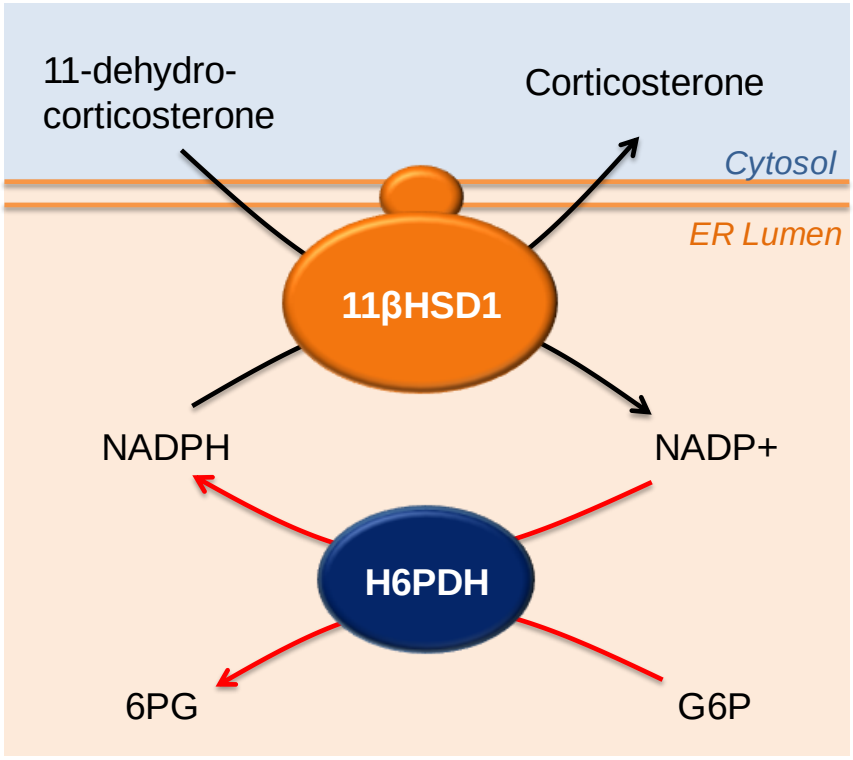

Figure 1.7 Reductase activity of 11βHSD1. In intact cells, 11βHSD1 functions as a reductase, converting inactive glucocorticoids, 11-dehydrocorticosterone in rodents, to the active form, corticosterone in rodents. For the reaction to proceed in this direction requires the presence of the cofactor nicotine adenine disphosphonucleotide (NADPH). The colocalised enzyme hexose-6-phosphate dehydrogenase (H6PDH) is responsible for converting glucose-6-phosphate (G6P) to 6-phospho-gluconolactone (6PG), thereby generating NADPH from $NADP^+$.

now established that 11βHSD1 provides an important intracellular amplification of glucocorticoids, as well as contributing to circulating levels of the hormone (Walker 2007). In particular, given the anti-inflammatory effects of glucocorticoids, increased expression of 11βHSD1 is evident during inflammation (Chapman *et al.* 2009). 11βHSD type 2 (11βHSD2) is the $NADP^+$-dependent isozyme that functions in the dehydrogenase direction, inactivating glucocorticoids (Morton and Seckl 2008). The expression pattern of 11βHSD2 is distinct in that it is abundantly expressed in mineralocorticoid target tissues such as the distal nephron, salivary glands and colonic mucosa. This is important as 11βHSD2 protects MR from inappropriate activation by glucocorticoids, which can activate mineralocorticoid pathways, causing sodium and water retention, leading to hypertension (Morton and Seckl 2008). Consistent with this, 11βHSD2 is often colocalised with MR at the endoplasmic reticulum membrane within cells (Odermatt *et al.* 2001).

1.2.7.2 5α-Reductases

5α-Reductases catalyse the reduction of $\Delta^{4,5}$ double bonds in a number of steroid substrates, yielding 5α-dihydro metabolites. Two isozymes exist, type 1 and type 2, which are the products of two different genes, *SRD5A1* and *SRD5A2* respectively (Andersson and Russell 1990; Andersson *et al.* 1991). Both the tissue distribution and substrate affinities differ between the two isozymes. In humans, 5αR1 (type 1) predominates in liver, adipose tissue, brain and non-genital skin, whilst 5αR2 (type 2) is the principal isozyme in male reproductive tissues such as the prostate, epididymis and seminal vesicle (Doering *et al.* 2002). In rodents, it has been shown that 5αR1 has a much greater affinity (10-20 fold higher) for corticosterone, androgens and progesterones than 5αR2 (Normington and Russell 1992). Tissue specific distribution has important physiological implications. 5αR2 is crucial for normal development of human male sexual characteristics, as shown in conditions of male pseudohermaphroditism (Andersson *et al.* 1991). This condition, characterised by normal development of urogenital tracts, but delayed development of external genitalia in males, is a result of a mutation in 5αR2, which converts testosterone to its more potent metabolite, 5α-dihydrotestosterone (5αDHT). 5α-Reduction of

steroids renders their 3-oxo groups more susceptible to reduction by 3α-HSD. Such modifications reduce the affinity of the steroid for binding proteins, and so it was believed that 5αR1 participated in the catabolism and inactivation of steroid substances (Carlstedt-Duke *et al.* 1977). However, following the finding that 5αDHT is a more potent steroid at the androgen receptor than testosterone (Siiteri and Wilson 1974), further investigations revealed that 5α-reduced metabolites of progesterone (Kenyon 1985), aldosterone (Smith *et al.* 1998), and more recently glucocorticoid (McInnes *et al.* 2004), also activate their cognate receptors .

1.2.7.3 5β-Reductase

Similar to 5α-reductases, 5β-reductase (5βR) catalyses the reduction of the $\Delta^{4,5}$ double bond within the A-ring of steroids. However, unlike 5α-reductases, this yields *cis* 5β-dihydro steroids. Expression of 5βR is mainly restricted to the liver, with small amounts detected in kidney tissues (Okuda and Okuda 1984). The predominant function of 5βR involves bile acid metabolism within the liver, as demonstrated by congenital defects in this metabolism pathway resulting from mutations in 5βR (Palermo *et al.* 2008). However, in terms of steroid metabolism, it is believed that 5βR participates in catabolism of steroids, with 5βR metabolites thought to be biologically inactive as they fail to activate classic steroid receptors (McInnes *et al.* 2004). However, it should be noted that 5β-reduced steroids are effective SXR activators (Blumberg *et al.* 1998).

1.2.7.4 3α-Hydroxysteroid dehydrogenases

Following 5α- or 5β-reduction of steroids, 3αHSDs rapidly reduce these 5α- or 5β-dihydro metabolites, forming 5α- or 5β-tetrahydro steroids. In the rat, only one isozyme has been identified and cloned (*akr1c9*) (Lin *et al.* 1999). In humans, four isozymes have been cloned from four different genes, namely 3αHSD type 1 (*AKR1C4*), 3α(17β)HSD type 2 (*AKR1C3*), 3αHSD type 3 (*AKR1C2*) and 20α(3α)-HSD (*AKR1C1*) (Penning 1999). Distinct tissue distribution of these isozymes is apparent in humans, with both brain and liver expressing all four, lung tissue

expressing all except 3αHSD type 1 and prostate predominantly expressing 3αHSD type 3 and 3α(17β)HSD type 2 (Penning 1999). 3αHSDs are believed to participate in catabolism of steroids, for example in human liver, 3αHSD type 1 reduces the active 5αDHT to the inactive 5α-tetrahydro form (Khanna *et al.* 1995). However, 3αHSDs function *in vivo* in both directions, dependent on the availability and ratio of $NADP^+$/NADPH. In human prostate, 3αHSD type 3 acts in the reverse direction as an oxidative enzyme, generating the active 5αDHT (Penning 1999). Therefore, the direction and activity of 3αHSD may critically influence the availability of active 5α-steroids.

1.2.8 Glucocorticoids in metabolic syndrome

The potential roles of glucocorticoid signalling in obesity and the associated metabolic syndrome are shown in Figure 1.8. The importance of glucocorticoids is exemplified in the clinical settings of deficiency, namely Addison's disease (Kyriazopoulou 2007), and excess, namely Cushing's syndrome (Boscaro *et al.* 2001). Addison's disease is characterised by hypotension, weight loss and hypoglycaemia, whereas patients suffering from Cushing's syndrome display hypertension, central obesity and hyperglycaemia. The striking similarities between glucocorticoid excess and the metabolic syndrome led to investigation into whether altered levels of glucocorticoids was responsible for the development of insulin resistance. Initial studies in idiopathic obesity revealed that whilst cortisol secretion is increased compared to lean subjects, circulating levels of glucocorticoids are unchanged due to enhanced metabolic clearance (Walker 2007). However, the discovery of intracellular metabolism of glucocorticoids as a modulator of receptor activation invoked much research into the roles of the 11βHSDs and the A-ring reductases in this disorder.

Elevated intra-adipose levels of glucocorticoids have been proposed to be a major determinant of obesity and the metabolic syndrome (Seckl and Walker 2001; Walker and Andrew 2006; Cooper and Stewart 2009). The enzyme 11βHSD1 regenerates active glucocorticoid from its inert 11-keto metabolite, thereby providing an elegant

system through which local levels of glucocorticoids could be increased. As such, the levels of this enzyme were investigated in both genetic models of rodent obesity, as well as human obesity, revealing an increase in adipose expression of 11βHSD1 (Livingstone *et al.* 2000; Masuzaki *et al.* 2001; Rask *et al.* 2001; Lindsay *et al.* 2003; Wake *et al.* 2003). Subsequent transgenic rodent models have further demonstrated the potential role this enzyme plays in metabolic dysregulation. Adipose-specific overexpression of 11βHSD1 in mice results in elevated adipose levels of corticosterone alongside all the major features of the metabolic syndrome (Masuzaki *et al.* 2001). In contrast, mice with a targeted disruption of 11βHSD1 show improved glucose tolerance and insulin sensitivity when fed a high-fat diet (Kotelevtsev *et al.* 1997; Morton *et al.* 2001; Morton *et al.* 2004). However, the detrimental effect of enhanced 11βHSD1 expression is not solely in adipose. Transgenic overexpression of 11βHSD1 in liver tissue also leads to the development of insulin resistance and dyslipidemia, but not obesity (Paterson *et al.* 2004).

Recent studies have suggested a possible link between the inflammation seen in obesity and the altered levels of 11βHSD1, with pro-inflammatory cytokines shown to increase expression and activity of 11βHSD1 in primary adipocytes (Tomlinson *et al.* 2001; Tomlinson *et al.* 2004). This work provides a potentially crucial mechanistic insight into the dysregulation of glucocorticoid metabolism in obesity.

Given the association of increased 11βHSD1 expression in obese subjects, a compelling case is made for targeted inhibition of 11βHSD1 as a therapeutic strategy to combat the metabolic complications associated with obesity. Numerous 11βHSD1 inhibitors have been tested in rodent models of obesity and insulin resistance, with varying results. However, several groups have reported compounds that lower plasma glucose and insulin levels in both diet-induced and genetic models of obesity (Alberts *et al.* 2002; Alberts *et al.* 2003). Translation of these inhibitors into human patients has recently been undertaken, with the results providing much promise. In a randomised, placebo-controlled study, patients with type 2 diabetes who had

insufficient glycaemic control with metformin treatment were given the 11βHSD1 inhibitor INCB13739 (Rosenstock *et al.* 2010). Over 12 weeks, it was found that treatment with the inhibitor improved hyperglycaemia in a dose-dependent manner, indicating that 11βHSD1 is a valid therapeutic target for the treatment of the metabolic syndrome.

Given that glucocorticoid secretion is increased in obesity, yet circulating levels are unchanged, it raises the question of whether there are alterations in the clearance mechanisms of glucocorticoids in obesity. Hepatic levels of 11βHSD1 are reduced in obesity, both in rodent models and in human subjects, lowering the regeneration of active glucocorticoids (Morton and Seckl 2008). However, the main route of glucocorticoid clearance is believed to involve A-ring reduction (Andrew *et al.* 1998; Walker and Andrew 2006; Morton and Seckl 2008). Investigation into the levels of A-ring reductases revealed that hepatic levels of 5αR1 are increased in obese humans and rodents, resulting in increased clearance of glucocorticoids, as demonstrated by increased excretion of 5α-reduced metabolites (Andrew *et al.* 1998; Livingstone *et al.* 2005). Given the detrimental effects that glucocorticoids have on insulin sensitivity, it would appear that increasing the clearance of these steroids could be an adaptive response employed by the liver to protect it from these effects. Support for this theory is shown by recent studies in 5αR1 knockout mice, which develop fatty liver and glucose intolerance, possibly due to accumulation of the parent glucocorticoid (Livingstone 2008). However, one might speculate that the recent discovery that 5α-reduced metabolites of glucocorticoids are able to activate GR alters this theory. As such, it became important to characterise these 5α-reduced metabolites in terms of their biological activity.

Work looking at the binding affinities of A-ring reduced metabolites of corticosterone for GR, as well as the level of receptor activation, found that 5α-reduced glucocorticoid metabolites are capable of displacing dexamethasone in competitive binding studies (McInnes *et al.* 2004). Recent work has shown that

whilst 5αTHB was unable to transactivate the gluconeogenic genes TAT and PEPCK, it was able to suppress the release of pro-inflammatory cytokines IL-6 and TNF-α in lipopolysaccharide (LPS) treated bone marrow derived macrophages, although to a much lesser extent than corticosterone (Yang 2009). This evidence suggests that 5αTHB is capable of retaining the anti-inflammatory properties of the parent glucocorticoid, without inducing the adverse metabolic effects. This lends support to the theory that increased 5αR1 expression in obesity is a protective mechanism, suppressing inflammation.

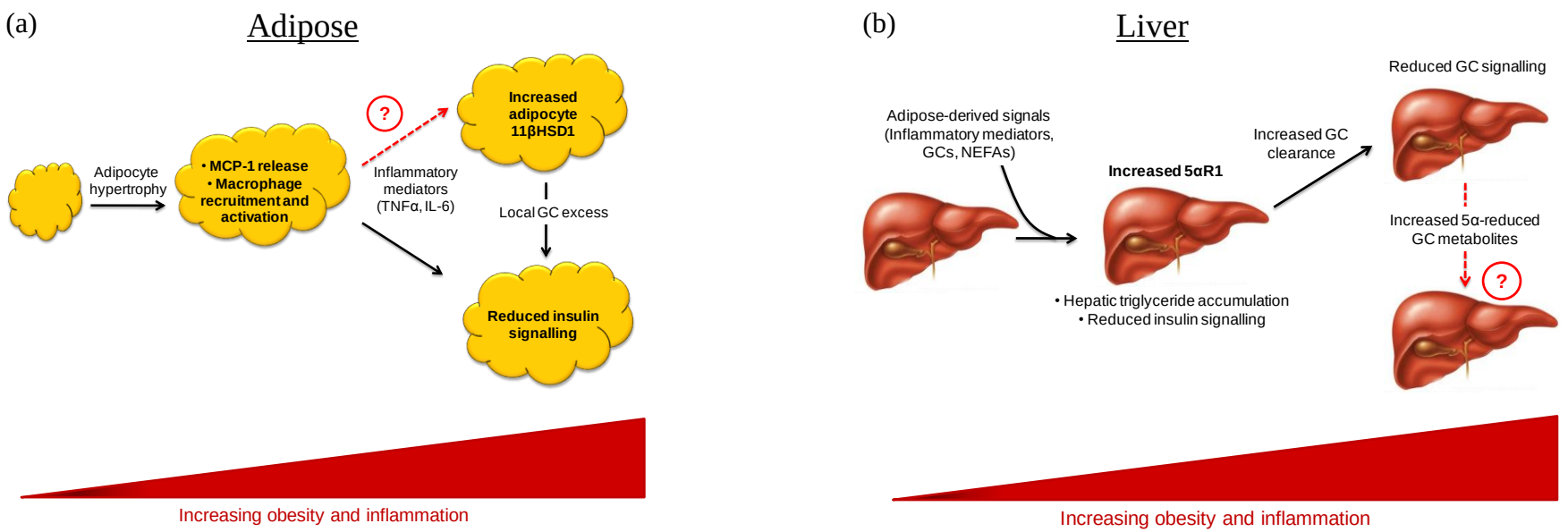

Figure 1.8 Overview of signalling in obesity. In adipose tissue (a), obesity is associated with adipocyte hypertrophy. This stimulates secretion of monocyte chemoattractant protein-1 (MCP-1), which results in the macrophage recruitment. Subsequent macrophage activation releases inflammatory mediators including tumor necrosis factor-α (TNF α) and interleukin-6 (IL-6), which directly affect insulin signalling. Increased glucocorticoid (GC) levels, via increased regeneration through 11βHSD1, are also known to affect insulin signalling. This thesis will assess potential roles for inflammatory regulation of adipose 11βHSD1 (shown by red dotted line). In liver (b), obesity increases the flux of adipose-derived mediators, including non-esterified fatty acids (NEFAs) to this tissue. this is associated with increased hepatic triglyceride accumulation, reduced insulin signalling and increased levels of the enzyme 5αR1, which reduces GCs into their 5α-metabolites. This altered GC metabolism has initially been viewed as a protective pathway, limiting the metabolic effects of GCs on the liver. However, the full range of actions of 5α-reduced GC metabolites has yet to be explored, and so effects on the liver are not fully understood (red dotted line). This thesis will explore the potential anti-inflammatory actions of 5α-reduced GC metabolites.

1.3 Hypotheses

Given that glucocorticoid metabolism is altered in obesity and associated insulin resistance and might influence local glucocorticoid levels and hence inflammation, and that inflammation plays a prominent role in the development of these metabolic disorders and is implicated in regulation of 11β-HSD1, it is possible that these events may regulate each other, contributing to the overall disease state. The hypotheses of this thesis are outlined below:

1) Salicylate treatment alters glucocorticoid metabolism, suppressing 11β-HSD1 in adipose tissue.

2) Suppression of adipose 11βHSD1 mediates the insulin-sensitising effects of anti-inflammatory salicylates in obesity.

3) 5α-Reduced glucocorticoid metabolites exert anti-inflammatory effects in a similar manner to the parent glucocorticoid.

4) 5α-Reduced glucocorticoid metabolites suppress inflammation *in vivo*.

Chapter 2

Materials and Methods

2.1 Materials

All chemicals and reagents were from Sigma-Aldrich, Dorset, UK, unless otherwise stated. All steroids were from Steraloids, RI, USA. Room temperature (RT) is defined as 18-22°C.

2.2 Buffers and Solutions

2.2.1 Molecular biology

2.2.1.1 DEPC-treated water

Diethylpyrocarbonate (DEPC; 5 drops) was added to distilled water (500ml), mixed, left for 24 hours prior to autoclaving and stored at RT.

2.2.1.2 10xTBE Buffer

Tris base (890mM), boric acid (890mM) and EDTA (0.5M, 40ml) were dissolved in distilled water (800ml). The pH was adjusted to 8.0 through addition of KOH (10M) and volume adjusted to 1L with distilled water. The solution was autoclaved and stored at RT.

2.2.1.3. 0.5xTBE Buffer

10xTBE (50ml) was diluted in distilled water (950ml) and stored at RT.

2.2.1.4 Protein Lysis Buffer (2x)

4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, 1M, pH 7.5, 50ml), NaCl (137mM), $MgCl_2$ (1mM), $CaCl_2$ (1mM), NaF (10mM), EDTA (2mM) and sodium pyrophosphate (10mM) were dissolved in distilled water (1L) and stored at RT.

2.2.1.5 Protein Lysis Buffer (1x)

Octylphenoxypolyethoxyethanol (IGEPAL CA-630, 1% v/v), glycerol (l0% v/v), $NaVO_4$ (2mM), protease inhibitor cocktail tablet (Roche, West Sussex, UK; x1) and lysis buffer (2x, 5ml) were added to distilled water (2.8ml). This was prepared immediately prior to use.

2.2.1.6 Running Buffer (10x)

Tris base (250mM), sodium dodecyl sulphate (SDS, 35mM) and HEPES (1M) were dissolved in distilled water (1L) and stored at RT. A working solution was used at 1x concentration.

2.2.1.7 Transfer Buffer

Tris base (25mM) and glycine (186mM) were dissolved in distilled water (900ml), before addition of methanol (100ml) and stored at 4°C.

2.2.1.8 TBS (10x)

Tris base (250mM), KCl (27mM) and NaCl (1.5M) were dissolved in distilled water (800ml). The pH was adjusted to 7.4 with concentrated HCl (37%), before the volume was adjusted to 1L with distilled water and stored at RT.

2.2.1.9 TBST (1x)

10xTBS (100ml) was diluted in distilled water (900ml), with the addition of Tween-20 (1ml) and stored at RT.

2.2.1.10 Blocking Milk (5%)

Blotting grade blocker, non-fat, dry milk (Biorad, CA, USA, 5g) was dissolved in 1x TBST (100ml) and stored at 4°C.

2.2.1.11 BSA (5%)

Bovine serum albumin, (BSA, Fraction V, 5g) was dissolved in TBST (1x, 100ml) and stored at 4°C.

2.2.1.12 KREBS Buffer

$MgSO_4.7H_2O$ (1.19mM), KCl (3.80mM), KH_2PO_4 (1.19mM), $CaCl_2$ (2.54mM), $NaHCO_3$ (25mM) and NaCl (118mM) were added to distilled water (76ml). The pH was adjusted to 7.4 with concentrated HCl (37%) and the solution stored at 4°C.

2.2.1.13 Borate buffer

Boric acid (66mM), NaOH (33mM), BSA (Fraction V, 0.5% w/v) and concentrated HCl (1.75ml) were dissolved in distilled water (500ml) and stored at -20°C.

2.2.1.14 Luciferase lysis buffer

Tris phosphate (25mM, pH 7.8), dithiothreitol (2mM), Triton x100 (1% v/v) and glycerol (10% v/v) were added to distilled water (21.5ml) and stored at -20°C.

2.2.1.15 Luciferase assay buffer (2x)

Tricine (59mM), dithiothreitol (66mM), EDTA (0.2mM), $MgCO_3$ (2mM), $MgSO_4$ (0.5mM) and Coenzyme A (0.26mM) were added to distilled water (48ml). The pH was adjusted to 7.5 and the solution stored at -20°C.

2.2.1.16 Luria Bertani (LB) broth

LB (25g) was dissolved in distilled water (1L).

2.2.1.17 LB/ ampicillin agar

Agarose (1.5g, Lonza, Berkshire, UK) was dissolved in LB broth (100ml, 2.2.1.16), before addition of ampicillin (100μg/ml).

2.3 Cultured cells

All cells were from the European Collection of Cell Cultures (ECACC), unless otherwise stated. All reagents for cell culture were from Lonza, Berkshire, UK, unless otherwise stated.

2.3.1 HEK293 (human embryonic kidney cells)

HEK293 cells were cultured in 75cm^2 flasks in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% (v/v) heat-inactivated foetal bovine serum (FBS), penicillin (100 IU/ml), streptomycin (100μg/ml) and L-glutamine (200mM). Cells were incubated in a humidified atmosphere in 95% air and 5% CO_2 at 37°C.

Cells were routinely passaged when confluent, usually twice a week. For subculturing, cells were washed with sterile phosphate buffered saline (PBS, 7ml) and then cells incubated with trypsin/EDTA (2ml, 1-2 min, 37°C). The flasks were shaken until cells were released from the flask surface. Cells were resuspended in medium (10ml). 1/10 to 1/4 of cells were left for maintenance in the flasks, and the rest were aspirated or transferred to other containers. The medium (12ml final volume) was added to the flasks before cells were incubated in the conditions described above, unless otherwise stated.

2.3.2 RAW264.7 (murine macrophage cells)

RAW264.7 cells were cultured 75cm^2 flasks under the same conditions as those described for HEK293 cells (2.7.1.1), however subculturing was slightly different. Cells were washed with sterile PBS (7ml) and fresh culture media added (9ml). Cells were detached from the surface of the flask and resuspended in normal culture media using a sterile scrapper. The remaining subculture protocol was as described for HEK293 cells (2.4.1).

2.4 *In vitro* experimental procedures

2.4.1 Luciferase reporter plasmids

The luciferase reporter plasmids 3x(NFκB)tk-luc and MMP1-luc (-517/+63col-luc) were kind gifts from L.G. Bladh (Karolinska Institutet, Sweden) and have been described previously (Bladh *et al.* 2005). The plasmids pGEM (inert DNA) and pGL3-basic (empty vector) were purchased from Promega (Hampshire, UK). The plasmids pSV2-luc (positive luciferase control) and pVL342 (glucocorticoid receptor) were kindly supplied by Prof. Karen Chapman (University of Edinburgh).

2.4.2 Plasmid preparation

The plasmids described above (2.8.1) were grown in *Escherichia coli* HB101 cells. A single colony of cells were grown overnight (37°C) in LB broth (5ml, 2.2.1.16). This overnight culture of cells was diluted 1/100 into LB broth (50ml) and incubated (2 hours, 37°C). Cells underwent centrifugation (6000g, 5 min, 4°C). The pellet formed was resuspended in calcium chloride (20ml, 0.1M) and incubated (10 min, 4°C). The

cells underwent further centrifugation (6000g, 5 min, 4°C), and the pellet formed was resuspended in calcium chloride (20ml, 0.1M) and incubated (4°C).

For each plasmid, DNA (1µl) was added to HB101 cells (200µl) and incubated (30 min, 4°C). Cells were heat-shocked (50 sec, 42°C) and incubated (2 min, 4°C), before being plated out on LB/ ampicillin agar plates (2.2.1.17) and incubated (overnight, 37°C). A single colony was selected and grown in LB broth with ampicillin (overnight, 37°C).

Plasmid purification was carried out using the Plasmid Maxi Kit (Qiagen). Overnight cultures were subjected to centrifugation (8000g, 10 min, 4°C), with the pellet formed resuspended thoroughly in Buffer P1 (10ml). Addition of Buffer P2 (10ml) and thorough mixing ensured lysis of cells. Following incubation (10 min, RT), Buffer P3 (10ml) was added, mixed thoroughly and incubated (10 min, 4°C) to precipitate out SDS. The suspension was subjected to centrifugation (16000g, 30 min, 4°C) and the supernatant added to a QIAGEN-tip, where DNA binds to the column. The QIAGEN-tip was washed twice with Buffer QC (30ml each), before DNA was eluted with Buffer QF (15ml). DNA was precipitated out by addition of 0.7x volume (10.5ml) isopropanol and subjection to centrifugation (15000g, 30 min, 4°C). The resulting pellet was washed with ethanol (5ml, 70% v/v) and subjected to centrifugation (15000g, 10 min, 4°C). The pellet formed was air-dried (5 min) and resuspended in distilled water (500µl), before DNA was quantified using a Nanodrop Spectrophotometer as described for RNA (2.6.1.4), with the exception that the ratio was deemed acceptable if between 1.7 and 1.9.. Plasmid DNA was stored (-20°C) until needed.

2.4.3 Transfection of cells

Cells were seeded on 6-well plates at a concentration of approximately 2-3 x 10^5 cells/ml and incubated for 24 hours. For each well to be transfected, the following transfection complex was made up:

- Plasmid DNA (1.2µg, outlined below)
- Opti-MEM Reduced Serum Medium (50µl, GIBCO, Parsley, UK)
- FuGENE HD Transfection Reagent (2µl, Roche, West Sussex, UK)

This transfection complex was incubated (15 min, RT), before addition to appropriate wells. Plasmid DNA was setup as outlined in Table 2.1. The plasmid pGEM, containing inert DNA, was used to as a negative control. The plasmid pSV2-luc was used as a positive control to confirm the success of each batch of transfection. The plasmid pGL3-basic was an empty vector control which was a luciferase reporter vector without promoter or enhancer. When the luciferase activity of cells transfected with pSV2-luc was >500 times higher than that of pGL3-basic, the transfection was deemed successful.

2.4.4 Cell lysate preparation

The growth medium from cells was removed, and then cells were rinsed carefully with sterile PBS (1ml). Lysis buffer (2.2.1.14, 100µl per well) was added and the culture plates rocked to ensure complete coverage of the cells with lysis buffer. The cells were then scraped from wells and transferred to an eppendorf tube prior to centrifugation (12000g, 2 min, RT) to precipitate any remaining undissolved cell lysate. The transparent supernatant was used in subsequent assays of luciferase.

Plasmid (μg)	Transfection						
	pGEM	pSV2	pGL3	With GR		Without GR	
				NFκB	AP-1	NFκB	AP-1
pGEM	1.2	-	-	-	-	-	-
pSV2-luc	-	1.2	-	-	-	-	-
pGL3-basic	-	-	1.2	-	-	-	-
3x(NFκB)tk-luc	-	-	-	1	-	1.2	-
-517/+63col-luc	-	-	-	-	1	-	1.2
pVL342	-	-	-	0.2	0.2	-	-

Table 2.1 Plasmid DNA composition and weights for transfection.

2.4.5 Luciferase assay

Luciferase activity was quantified on a 96-well plate (Nunclon Surface, Denmark). The following were added to each well: sample (duplicate; 40µl), assay buffer (2.2.1.15; 100µl) and ATP (0.1M; 5µl). The plate was placed into a Microplate Luminometer (Orion II, Berthold, Hertfordshire, UK), in which luciferin (1mM; 100µl) was injected to each well. The reaction between the substrate luciferin and co-substrate ATP Mg^{2+} was catalyzed by the firefly luciferase when present within the cells, forming the product of oxyluciferin. During the oxidation of luciferin, light was produced by converting the chemical energy derived from an electron transition. Using the programme Simplicity 4.1 (Berthold, Hertfordshire, UK), the luminescence from each well was read with a 2.05 sec delay after injection, and each measurement lasted for 10 sec. When the luciferase activity of cells transfected with pSV2-luc was 500 times higher than that of pGL3-basic, the transfection was deemed successful. Activity from pGEM was subtracted from all values as a measure of background activity. Duplicates were acceptable if they differed from mean <10%.

2.5 *In vivo* procedures

2.5.1 Animal Maintenance

Animals were maintained under controlled conditions of light (lights on 0700h – 1900h) and temperature (18-20°C) and allowed free access to standard chow (RMI 801002; Special Diet Services, Witham, UK) and drinking water, unless otherwise stated. All experiments were performed under the following project licence (JR Seckl, PPL No: 60/3962) and personal licence (Nixon, PIL No: 60/11505) and under the guidelines of the UK Home Office.

2.5.2 Mini-pump Implantation

2.5.2.1 Mini-pump loading

Solutions of vehicle or drug were made up immediately prior to loading of mini-pumps. Osmotic mini-pumps (Model 2004, Alzet, CA, USA) were loaded with appropriate solution following manufacturer's instructions and primed in saline at 37°C for 24 hours before surgery. These pumps perform due to differences in osmotic pressure between the loading compartment within the pump and the tissue environment in which the pump is implanted. This causes an influx of water into the area around the loading compartment, displacing the test solution from the pump at a controlled rate (0.25µl/hr).

2.5.2.2. Surgery

Animals were injected subcutaneously with carprofen (5mg/kg body weight, 1 in 10 diluted in saline (0.9% w/v, B.Braun Melsungen AG, Germany)) and anaesthetised with isoflurane (Merial Animal Health Ltd, Essex, UK). Loaded osmotic mini-pumps were implanted subcutaneously between the scapulae through dorsal incisions which were closed with staples. Following surgery, animals were allowed to recover on a heat mat before being transferred to individual clean cages.

2.5.3 Thioglycollate-induced peritonitis

Thioglycollate (0.5ml, 10% v/v in distilled water) was administered by intraperitoneal injection at 1100h. Animals were decapitated 4 hours after induction of peritonitis (1500h) and within 1 min of disturbing the cage. Trunk blood was collected in ETDA-treated tubes (0.5M). Plasma was prepared from blood samples by centrifugation (10000g, 5 min, 4°C) and stored at -20C. Peritoneal lavage was performed immediately after decapitation with sterile PBS (4ml). Lavage fluid was stored (4°C) until quantification by flow cytometry.

2.5.4 Glucose tolerance test

Mice were fasted for 6 hours (0800h – 1400h) before initiation of glucose tolerance test. Glucose (40% w/v in distilled water) was administered by intraperitoneal injection to give a dose of 2g glucose per kg body weight. Blood was collected via tail nick before glucose injection and at 15, 30, 60 and 90 min post-injection. Following this procedure, animals were transferred to clean cages with free access to food and water.

2.5.5 Terminal procedures

Animals were decapitated on the morning (0800h – 1000h) of final day within 1 min of disturbing the cage. Trunk blood was collected in EDTA (0.5M)-treated tubes. Plasma was and stored as above (2.5.3). The following tissues were removed, weighed and snap frozen on dry ice; thymus, spleen, liver, kidney, adrenal, omental adipose, mesenteric adipose, retroperitoneal adipose, epididymal adipose and subcutaneous adipose. Tissues were then stored at -80°C.

2.6 Molecular Biology

2.6.1 RNA

All kits for RNA extraction and reverse transcription were from Qiagen, West Sussex, UK.

2.6.1.1 RNA extraction from liver

RNA was extracted from liver using the Qiagen RNeasy Mini Kit. Tissue (~30mg) was homogenised in Buffer RLT (600µl). The samples were subjected to

centrifugation (16,000g, 3 min, 4ºC). The supernatant was removed into an equal volume of 50% ethanol and mixed. This solution was placed in the RNeasy spin column and subjected to centrifugation (12,000g, 30sec, 4ºC). The eluate was discarded and the column washed with Buffer RW1 (700µl) and Buffer RPE (500µl) sequentially. In each case, the eluate was discarded following centrifugation (12,000g, 30 sec, 4ºC). Buffer RPE (500µl) was added to wash the membrane, followed by a further centrifugation step (12,000g, 2 min, 4ºC). The spin column was then placed in a fresh 2ml collecting tube and subjected to centrifugation (16,000g, 1 min, 4ºC) to eliminate any Buffer RPE carryover. The spin column was then placed in a fresh 1.5ml eppendorf, RNase-free water (30µl) added and RNA eluted by centrifugation (12,000g, 1 min, 4ºC). Eluted RNA was stored at -80°C.

2.6.1.2 RNA extraction from adipose

RNA was extracted from adipose using the same method as with liver, except for tissue homogenisation. Tissue (~30mg) was homogenised in Qiazol Lysis Reagent (600µl, Qiagen) and incubated (5 min, RT). Chloroform (200µl) was added, mixed and subjected to centrifugation (12,000g, 15 min, 4˚C). The supernatant was removed into an equal volume of ethanol (70% v/v) and mixed. At this stage, the solution was placed in the RNeasy spin column, with the remainder of the procedure being the same as for liver tissue shown above (2.6.1.1).

2.6.1.3 RNA extraction from cultured cells

RNA was extracted from cells using the Qiagen RNeasy Mini Kit. Cells were washed in sterile PBS (1ml) before addition of Buffer RLT (350µl per well) to lyse the cells. Cells were incubated (15 min, 4°C) and then scraped into eppendorfs. Lysates were transferred to the QIAshredder spin column and subjected to centrifugation (16000g, 2 min, 4ºC). The supernatant was removed into an equal volume of 70% ethanol and mixed. At this stage, the solution was placed in the RNeasy spin column, with the remainder of the procedure being the same as for liver tissue (2.6.1.1).

2.6.1.4 RNA quantification

RNA was quantified using a Nanodrop Spectrophotometer (Thermo Fisher, West Sussex, UK). Concentration was determined by the absorbance at 260nm wavelength (A260), and the purity assessed by the ratio of RNA/DNA (A260/A280), which was deemed acceptable if between 1.9 and 2.1.

2.6.1.5 RNA quality

Quality of RNA was assessed by electrophoresis on a denaturing agarose (Lonza, Berkshire, UK) gel (1.2% w/v in 0.5x TBE). Samples (2µl) were prepared by adding loading dye (Promega, WI, USA; 1 in 5 diluted in distilled water; 10µl). Prepared samples (12µl) were electrophorosed on the gel (100V, 1hr) and RNA integrity assessed on basis of 18S and 28S ribosomal RNA (rRNA) bands. RNA integrity was deemed satisfactory if sharp, clear 28S and 18S rRNA bands were present without smearing, and if 28S rRNA band was approximately twice as intense as 18S rRNA band.

2.6.1.6 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Total RNA (liver 1µg, adipose 0.25µg) was reverse transcribed using the QuantiTect Reverse Transcription Kit. RNA (12µl) was added to gDNA Wipeout Buffer (2µl) and incubated (42ºC, 2 min) to eliminate any contaminating genomic DNA. To each sample, Quantiscript RT Buffer (4µl, containing dNTPs), RT Primer Mix (1µl, containing oligo-dT and random primers) and Quantiscript Reverse Transcriptase (6µl) were added. Two negative controls were prepared as above, one with water instead of RNA to identify any RNA contamination in reagents and one without the reverse transcriptase enzyme in order to detect the presence of contamination by genomic DNA. Samples were incubated (42ºC, 15 min; 95ºC, 3 min) in a PCR machine (G-Storm, GRI Lid, Essex, UK), before being chilled to 4ºC. The resultant cDNA was stored at -20ºC.

2.6.1.7 Polymerase Chain Reaction (PCR)

PCR was carried out using Qiagen Multiplex PCR Kit. cDNA samples (1µl) were added to Qiagen water (7µl), primer mix (containing forward and reverse primers (Table 2.1), 2pmoles/µl for each primer, 2µl) and Qiagen Mastermix (10µl). A negative control was prepared with Qiagen water added in place of cDNA to confirm absence of DNA contamination. Thermal cycling was performed in a PCR machine (G-Storm, GRI Lid, Essex, UK). Samples were heated for initial denaturation (95°C, 5 min), then underwent 30 cycles of PCR amplification, which consisted of denaturation (95°C, 30 sec), primer annealing (55°C, 90 sec) and elongation (72°C, 60 sec). Upon completion of the PCR programme, samples were incubated to ensure elongation of products to full length (72°C, 5 min) and chilled to 10 °C. Cresol red loading dye (4mg/ml cresol red in 40% w/v sucrose; 5µl) was added to each sample, mixed and analysed by electrophoresis on an agarose (Lonza, Berkshire, UK) gel (1.2% w/v in 0.5x TBE). A 100 base pair (bp) DNA ladder (Promega, WI, USA; range 100 – 1000bp) was also included to allow identification of products based on bp number (Table 2.2)

Gene Accession No.	Primer sequence		Product (bp)
Srd5a1 (5α-reductase 1) NM_175283	For	CTA CAG GAG CTG CCT TCA AT	122
	Rev	CTT TGC ACG TAG TGG ATC AG	
Srd5a2 (5α-reductase 2) NM_053188	For	AAC ACA GCG AGA GTG TGT CG	160
	Rev	GAG AAG AGA CCC AGC AGC AC	
Akr1d1 (5β-reductase) NM_145364	For	ATG CCG CCT ATG TTT ACC AC	500
	Rev	ATG TGC GAC AAT GAC GAT GT	
Akr1c6 (3α-HSD) NM_030611	For	AAT TGG TCC GAT CTT GCT TG	408
	Rev	CCA CCC AGA TTT TGT CTC GT	
Gapdh (GAPDH) NM_008084.2	For	ATG GTG AAG GTC GGT GTG AAC	258
	Rev	GCC TTG ACT GTG CCG TTG AAT	

Table 2.2 Details of primers for PCR amplification (For, forward; Rev, reverse; bp, base pair).

2.6.1.8 Real-time PCR

Abundance of mRNA was quantified using a LightCycler 480 (Roche Applied Science, Burgess Hill, UK). Primers (Table 2.3; Invitrogen Ltd., Paisley, UK) were designed to match intron-spanning probes within the Roche Universal Probe Library. cDNA was diluted (liver, 1 in 40; adipose, 1 in 20; cultured cells, 1 in 20) in LightCycler H_2O. Diluted cDNA samples (2µl) were added to each well, followed by Master Mix (8µl), consisting of the following components:

LightCycler H_2O (2.7µl)

Forward and reverse primers (20µM, 0.1µl each)

Corresponding probe (10µM, 0.1µl)

Roche Probes Master (5µl)

Samples were heated for initial denaturation (95ºC, 5min), then underwent 50 cycles of PCR amplification, which consisted of denaturation (95ºC, 10 sec), annealing (60ºC, 30 sec) and elongation (72ºC, 1 sec). Upon completion of the PCR programme, samples were cooled (40ºC, 30 sec). All samples were analysed in triplicate and amplification curves plotted (y axis fluorescence, x axis cycle number). Triplicates were deemed acceptable if the standard deviation of crossing point (Cp) < 0.5 cycles. A standard curve (y axis crossing point, x axis log concentration) for each gene was generated by serial dilution of cDNAs pooled from different samples and fitted with a straight line and deemed acceptable if reaction efficiency was between 1.7 and 2.1.

Gene Accession No.	Primer sequence		UPL Probe No.
Hsd11b1 (11βHSD1) NM_008288.2	For	TCT ACA AAT GAA GAG TTC AGA CCA G	1
	Rev	GCC CCA GTG ACA ATC ACT TT	
Srd5a1 (5α-reductase 1) NM_175283	For	GGG AAA CTG GAT ACA AAA TAC CC	41
	Rev	CCA CGA GCT CCC CAA AAT A	
Agt (Angiotensinogen) NM_007428.3	For	CGA CCT CCT GAC TTG GAT AGA	62
	Rev	AAA GGG TGG GCA GCT TGT	
Tnf (TNFα) NM_013693.2	For	CTG TAG CCC ACG TCG TAG C	25
	Rev	TTG AGA TCC ATG CCG TTG	
Adipoq (Adiponectin) NM_009605.4	For	GGA GAG AAA GGA GAT GCA GGT	17
	Rev	CTT TCC TGC CAG GGG TTC	
Lpl (Lipoprotein lipase) NM_008509.1	For	CTC GCT CTC AGA TGC CCT AC	95
	Rev	GGT TGT GTT GCT TGC CAT T	
Lip (Hormone sensitive lipase) NM_001039507.1	For	GCG CTG GAG GAG TGT TTT T	3
	Rev	CCG CTC TCC AGT TGA ACC	
Pnpla2 (Adipose triglyceride lipase) NM_025802.2	For	CCT CTC GAA GGC TCT CTT CC	67
	Rev	GGT CCT TTG GTT CCA CAC AG	
Rn18s (18S) NR_003278.1	For	CTC AAC ACG GGA AAC CTC AC	78
	Rev	CGC TCC ACC AAC TAA GAA CG	
Tbp (TATA-binding protein) NM_013684.3	For	GGG GAG CTG TGA TGT GAA GT	97
	Rev	CCA GGA AAT AAT TVT GGC TCA	

Table 2.3 Details of primers for real-time PCR for use with Roche Universal Probe Library (UPL) (for, forward; rev, reverse).

2.6.2 Protein

2.6.2.1 Protein extraction from tissue

Tissue (adipose, 60mg) was homogenised using a rotor-blade in KREBS (2.2.1.12; 1.8ml) subjected to centrifugation (1000g, 1 min, RT) to pellet cellular debris. The supernatant containing the protein was then harvested and stored at -20°C.

2.6.2.2 Protein extraction from cultured cells

Cells were washed with sterile PBS (1ml). Lysis buffer (2.2.1.5; 100µl per well) was added and incubated (15 min, 4°C). Cells were scraped into an eppendorf, mixed and further incubated (5 min, 4°C), before being subjected to centrifugation (13000g, 20 min, 4°C) to generate a pellet composed of heavy membranes. The supernatant containing cytosolic protein was stored at -20°C.

2.6.2.3 Protein assay

Protein concentrations of samples were determined using a Bio-Rad kit (Bio-Rad Laboratories Ltd., Hertfordshire, UK). The absorbance maximum for the acidic solution of Coomassie® Brilliant Blue G-250 dye shifts from 465nm to 590nm when binding to protein occurs. The absorbance at 590mn is proportional to the concentration of protein present. Standards were prepared from a stock solution in KREBS (2.2.1.12; adipose) or protein lysis buffer (2.2.1.5; cultured cells). Samples (10µl) were added in duplicate to wells of a 96-well plate before addition of Biorad acidic dye reagent (1 in 5 dilution in distilled water; 240µl). Absorbance of samples and standards were recorded at 590nm. Data from the standards were used to construct standard curves (y axis absorbance, x axis concentration) which were deemed acceptable if r^2>0.98 and duplicates differed from mean < 10%.

2.6.2.4 Enzymology

11βHSD1 functions as a reductase *in vivo*, converting inactive 11-dehydrocorticosterone to corticosterone. However, *in vitro*, dehydrogenase activity predominates, so velocity of 11βHSD1 activity in adipose was quantified through conversion of corticosterone to 11-dehydrocorticosterone.

Enzyme kinetics were determined from whole tissue homogenate, with first order kinetics determined by ensuring conversion <30%. Adipose homogenates (100 or 300μg/ml) were incubated (37ºC) in KREBS buffer containing glucose (1% w/v), NADP (2mM), 1,2,6,7-[^{3}H]$_{4}$-corticosterone (10nM, GE Healthcare Life Sciences, Buckinghamshire, UK) and unlabelled corticosterone (adipose, 1.99 or 9.99μM). Steroids were extracted after a fixed period of incubation (adipose, 16 or 24 hours) with 10x volume ethyl acetate and the organic phase evaporated under oxygen free nitrogen at 60^{o}C. The dried extracts were then dissolved in mobile phase (600μl: 60% H_2O; 15% acetonitrile; 25% methanol). Steroids were injected using a 717plus Autosampler (Water, Hertfordshire, UK) and analysed by high performance liquid chromatography. Steroids were eluted (600 Controller pump, Waters, Hertfordshire, UK) with mobile phase (1.5ml/min, 45ºC) from a C_{18} Sunfire column (length 15cm; internal diameter, 4.6mm, pore size 5μm; Waters, Hertfordshire, UK) and quantified by on-line scintillation counting after mixing with Goldstar scintillation fluid (2ml/min; Meridian, Surrey, UK) using a scintillation pump (Berthold, Hertfordshire, UK). The proportion of corticosterone converted to 11-dehydrocorticosterone was calculated by integrating the peak areas of the analytes in the radiochromatograms using Chromeleon software (Dionex, CA, USA). Assays were performed in duplicate and deemed acceptable if differed from mean < 10%. Minimum peak height was set at 3x background.

2.6.2.5 Western blotting

2.6.2.5.1 Gel electrophoresis

Protein samples (30μg) were added to equal volume of loading mix, consisting of NuPAGE LDS sample loading dye (Invitrogen, Paisley, UK, 4x) and NuPAGE

sample reducing agent (Invitrogen, 1x). Samples were mixed and incubated (5 min, 95°C) to denature protein. Samples were separated by electrophoresis on a 12% Pierce Precise protein gel (Thermo Fisher Scientific, Northumberland, UK) using the XCell Surelock™ Mini-Cell (Invitrogen). The XCell tank was filled with 1x running buffer (2.2.1.6) and run at 80V for 1 hour at RT.

2.6.2.5.2 Gel transfer

Gels were transferred to nitrocellulose membranes (GE Healthcare, Buckinghamshire, UK) using the Mini Trans-Blot Electrophoretic Transfer Cell (Biorad, CA, USA). A gel sandwich was prepared within the cassette consisting of fiber pad, filter paper, gel, nitrocellulose membrane, filter paper and fiber pad. The gel sandwich cassette was placed within the transfer module and tank, which was then filled with transfer buffer (2.2.1.7). The transfer was run at 70V for 1 hour at 4°C.

2.6.2.5.3 Immunoblotting

After transfer, nitrocellulose membrane was blocked with milk (2.2.1.10, 1 hour, RT). The membrane was washed three times (10 min each) with TBST (2.2.1.9). The membrane was then incubated (overnight, 4°C) with primary antibody at a concentration of 1:1000 (unless otherwise stated) in BSA (2.2.1.11). The membrane was washed three times as before prior to incubation with secondary antibody at a concentration of 1:10000 (unless otherwise stated) in milk (2.2.1.10) for 1 hour. A final series of washes was performed as above, before the blot was developed.

2.6.2.5.4 Blot development

Blots were visualised using an Odyssey Imaging System (LI-COR Biosciences, Cambridgeshire, UK).

2.7 Biochemical assays

All spectrophotometric measurements were carried out with an OPTImax Tunable Microplate Reader (Molecular Devices, CA, USA).

2.7.1 Quantification of insulin in plasma by enzyme-linked immunosorbent assay (ELISA)

Insulin was quantified using an Ultra Sensitive Mouse Insulin ELISA Kit (Crystal Chem Inc., Illinois, USA). A series of dilutions of insulin were prepared (0.1 – 6.4 ng/ml) from the standard using sample diluent according to the kit. Samples (singlicate due to paucity of sample; 5µl) and standards (duplicate; 5µl) were added to wells pre-coated with primary guinea pig anti-insulin antibody along with sample diluent (95µl). Sample diluent was used as a blank measurement. After incubation (2 hours, 4˚C), the plate was washed five times in wash buffer supplied (diluted 1 in 20 in distilled water) to remove unbound insulin. Horse-radish peroxidise (HRP)-conjugated anti-insulin antibody conjugant (100µl) was added to each well and incubated (30 min, RT). The plate was washed a further seven times using the diluted wash buffer to remove unbound excess HRP-conjugate before addition of enzyme substrate solution (100µl) containing tetramethylbenzidine (TMB) to each well. The plate was incubated in the dark (40 min, RT) before addition of sulphuric acid (0.5M, 100µl) to each well to stop the reaction. The difference in absorbance between 630nm and 450nm was measured spectrophotometerically (values at 630nm are deemed to be a result of scratches or artefacts on the plate and are therefore subtracted to give a more accurate reading). A standard curve was constructed (y axis absorbance, x axis concentration) and a straight line fitted and deemed acceptable if $r^2 > 0.98$. Duplicates of standards were deemed acceptable if they differed from mean < 10%.

2.7.2 Quantification of glucose in plasma by hexokinase assay

Glucose levels were quantified using an Infinity Glucose Hexokinase Liquid Stable Reagent (Thermo Electron, Pittsburgh, USA) based on the following reactions. The reagent supplied contained hexokinase, which catalysed the phosphorylation of glucose by ATP, producing ADP and glucose-6-phosphate. The latter was then oxidised to 6-phosphogluconate with the associated reduction of NAD^+ to NADH by glucose-6-phosphate dehydrogenase. The amount of NADH formed was proportional to the concentration of glucose in the sample.

Serial dilutions of glucose were prepared (50 – 400mg/dl) from the standard using distilled water, with distilled water used as a blank. Samples (singlicate due to paucity of sample; 2µl) and standards (duplicate; 2µl) were added to wells of 96-well plates, followed by addition of the reagent (200µl). The plate was incubated in the dark (15 min, RT). The absorbance at 340nm was measured spectrophotometrically. A standard curve was constructed (y axis absorbance, x axis concentration) and a straight line fitted and deemed acceptable if $r^2 > 0.98$. Duplicates of standards were deemed acceptable they differed from mean < 10%.

2.7.3 Quantification of non-esterified fatty acid (NEFA) in plasma

NEFAs were quantified using a 96-well Serum/Plasma Fatty Acid Kit (Zen-Bio, NC, USA) based on the following reactions. NEFAs and CoA were firstly converted to fatty acyl-CoA thiol esters, in the presence of acyl-CoA synthetase, which subsequently reacted with oxygen in the presence of acyl-CoA oxidase, producing hydrogen peroxide. Thereafter 3-methyl-N-ethyl-N-(β-hydroxyethyl) -aniline and 4-aminoantipyrine were oxidized by hydrogen peroxide in the presence of peroxidase and produced a purple product with an absorbance of 550 nm.

Serial dilutions of NEFAs were prepared (1.4 - 333µM) from a NEFA Standard using distilled water, with distilled water used as a blank. Samples (singlicate due to paucity of sample; 5µl) and standards (duplicate; 5µl) were added to wells of 96-well

plate, followed by Dilution Buffer (45µl). NEFA Reagent A (100µl) was added to each well and plate incubated (10min, 37C°). NEFA Reagent B (50µl) was added to each well and plate incubated (10min, 37C°). Following incubation, the plate was allowed to equilibrate (5min, RT) before absorbance was read at 540nm. A standard curve was constructed (y axis absorbance, x axis concentration) and a straight line fitted and deemed acceptable if $r^2 > 0.98$. Duplicates of standards were deemed acceptable if they differed from mean < 10%.

2.7.4 Quantification of triglycerides in plasma

Plasma triglyceride levels were assessed using Infinity Triglyceride Liquid Stable Reagent (Thermo Electron, Pittsburgh, USA) based on the following reactions. Triglycerides were hydrolysed to glycerol and NEFAs by lipoprotein lipase. Glycerol was phosphorylated by ATP with glycerol kinase, producing ADP and glycerol-3-phosphate. The latter is then oxidised by glycerolphosphatase to produce hydrogen peroxide. Hydrogen peroxide reacts with 4-aminoantipyrine and 3, 5-dichloro-2-hydroxybenzene sulfonate to produce a red coloured dye. The absorbance of this dye was proportional to the concentration of triglycerides in the sample.

Serial dilutions of triglyceride were prepared (0.565 – 11.3mmol/l) from the standard calibrator using distilled water, with distilled water used as a blank. Samples (singlicate due to paucity of sample; 2µl) and standards (duplicate; 2µl) were added to wells of 96-well plate, followed by addition of the reagent (200µl). The plate was incubated in dark (5 min, 37ºC) before the difference in absorbance was read between 600nm and 500nm. A standard curve was constructed (y axis absorbance, x axis concentration) and a straight line fitted and deemed acceptable if $r^2 > 0.98$. Duplicates of standards were deemed acceptable if they differed from mean < 10%.

2.7.5 Quantification of corticosterone in plasma by radioimmunoassay (RIA)

Plasma corticosterone was quantified using an in-house assay (Al-Dujaili *et al.* 1981). Plasma was diluted (10% v/v) with borate buffer (2.2.1.13) and incubated (30 min, 80˚C) to denature corticosteroid-binding globulin (CBG). Serial dilutions of corticosterone were prepared (0.6 – 320nM) from a standard using borate buffer. Sample and standards (20µl) were added to wells of 96-well plate. A mix of primary antibody-$^{3}[H]_{4}$-corticosterone was prepared as follows: $[^{3}H]_{4}$-corticosterone (3µl) was added to borate buffer (6ml), followed by addition of rabbit anti-mouse primary antibody (60µl, 1 in 100 dilution), a kind gift from Dr Christopher Kenyon, University of Edinburgh (MacPhee *et al.* 1989). Primary antibody-^{3}H-corticosterone mix (50µl) was added to each well. 50µl of SPA beads (GE Healthcare Life Sciences, Buckinghamshire, UK) coated with anti-rabbit secondary antibody were added to each well and the plate incubated in the dark (16 hours, RT). Scintillation occurred when the SPA beads bound to that fraction of primary antibody which was associated with $[^{3}H]_{4}$-corticosterone. Due to the competition between binding of unlabelled and labelled corticosterone to the primary antibody, the extent of scintillation decreased as the concentration of unlabelled corticosterone increases. The degree of scintillation was quantified using a liquid scintillation counter (Microbeta Plus, Wallac 1450, Turku, Finland), and data (Bound/Bound at zero) used to construct a semi-log standard curve from which the concentration of corticosterone in each sample was calculated. Standard curves were deemed acceptable if $r^2 > 0.98$. Duplicates of standards were deemed acceptable if they differed from mean < 10%.

2.7.6 Quantification of β-hydroxybutyrate in plasma

Plasma β-Hydroxybutyrate (β-HB) was analysed using a β-Hydroxybutyrate Assay Kit (BioVision, CA, USA) based on the following reaction. β-HB was converted to acetoacetate by the enzyme β-hydroxybutyrate dehydrogenase. This product has an absorbance that is read at 450nm.

Serial dilutions were prepared (1 – 20mM) from the β-Hydroxybutyrate Standard using distilled water. Samples (singlicate due to paucity of sample; 5µl) and standards (duplicate; 5µl) were added to wells of 96-well plate, followed by addition of Reaction Mix (50µl). The plate was incubated in dark (5 min, RT) before absorbance was read at 450nm. A standard curve was constructed (y axis absorbance, x axis concentration) and a straight line fitted and deemed acceptable if $r^2 > 0.98$. Duplicates of standards were deemed acceptable if they differed from mean < 10%.

2.7.7 Quantification of cytokines by ELISA

Cytokine levels in cell media was quantified using the ELISA Ready-SET-Go! Kit (eBioscience, Hatfield, UK). All reagents were supplied by the kit unless stated. The 96-well plates provided were coated with Capture Antibody (100µl per well) and incubated (overnight, 4°C). The plates were washed five times with Wash Buffer (250µl per well) to remove unbound antibody and then blocked with 1x Assay Diluent (200µl per well, 1 hour, RT). The plates were washed five times as above. A series of dilutions of the specific cytokine were prepared using 1x Assay diluents (IL-6, 4pg/ml – 500pg/ml; TNFα, 8pg/ml – 1000pg/ml), with 1x Assay diluent used as a blank. Samples and standards (duplicate, 100µl) were added and incubated (overnight, 4°C). Following incubation, the plates were washed five times as above, Detection Antibody added (100µl per well) and plates incubated (1 hour, RT). The plates were washed five times as above, Avidin-HRP solution added (100µl per well) and incubated (30 min, RT). The plates were washed seven times with Wash Buffer (250µl per well), Substrate Solution added (100µl) and incubated (15 min, RT), then stop solution was added (50µl per well). The change in absorbance between 570nm and 450nm was measured spectrophotometrically. A standard curve was fitted according to manufacturer's instructions and deemed acceptable if $r^2 > 0.98$. Duplicates were deemed acceptable if they differed from mean < 10%.

2.8 Statistics

Data are presented as mean ± SEM and were analysed by Student's t-tests, One-way Analysis of Variance (ANOVA), or Two-way ANOVA followed by post-hoc tests as appropriate. Statistical significance was taken at the 5% level. Calculations were performed using GraphPad Prism 4.0 software (San Diego, CA, USA).

Chapter 3

Effects of anti-inflammatory salicylates on glucocorticoid metabolism in obesity

3.1 Introduction

In recent years, a plethora of evidence has accumulated indicating that inflammation is a key component in the underlying mechanism through which obesity causes insulin resistance (Mathieu *et al.* 2010; Olefsky and Glass 2010). Obese adipose tissue is viewed as being in a state of chronic, low-grade inflammation, with the levels of the pro-inflammatory cytokines TNFα and IL-6 positively correlated with the degree of obesity-linked insulin resistance. It is therefore reasonable to hypothesise that suppressing inflammation in this tissue may be beneficial. Non-steroidal anti-inflammatory drugs, such as sodium salicylate and aspirin, are amongst the most widely used medications in the world for treatment of inflammatory conditions such as rheumatoid arthritis. These effects are mediated by blocking production of pro-inflammatory molecules through several mechanisms, namely inactivation of COX-1 and COX-2 (Loll *et al.* 1995) and the more recently discovered inhibition of NFκB-mediated transcription (Kopp and Ghosh 1994). However, despite the discovery over half a century ago of their beneficial effects in the treatment of hyperglycaemia, it was not until recently that the mechanisms behind these glucose-lowering abilities of salicylates were re-investigated. Both rodent and human data have demonstrated the ability of salicylate compounds such as sodium salicylate, acetylsalicylic acid (aspirin) and salsalate to lower blood glucose levels in obesity and type 2 diabetes (Kim *et al.* 2001; Yuan *et al.* 2001; Hundal *et al.* 2002; Mohlig *et al.* 2006; Fleischman *et al.* 2008; Goldfine *et al.* 2008; Koska *et al.* 2009; Goldfine *et al.* 2010). These studies have implicated inflammatory pathways, particularly the IKKβ/NFκB system as the target of these compounds. However, the precise mechanism remains to be elucidated.

The clinical similarities between the excessive glucocorticoid levels seen in Cushing's syndrome and the metabolic consequences of obesity and insulin resistance led to the belief that dysregulation of glucocorticoid action was central to the pathogenesis of the metabolic syndrome. It has since been discovered that the levels of the glucocorticoid-regenerating enzyme 11βHSD1 are altered in obesity. In human obesity, 11βHSD1 mRNA levels and activity are decreased in liver, but

increased in subcutaneous adipose tissue, resulting in elevated intra-adipose cortisol regeneration (Rask *et al.* 2001; Lindsay *et al.* 2003; Wake *et al.* 2003). In genetically obese rodents, 11βHSD1 is increased in visceral adipose (Masuzaki *et al.* 2001; Livingstone *et al.* 2005), whilst adipose-specific overexpression of 11βHSD1 results in increased glucocorticoid action and recapitulates the obese and insulin resistance state (Paterson *et al.* 2004). In contrast, targeted disruption of *Hsd11b1* protects against insulin resistance and central obesity on high fat feeding (Kotelevtsev *et al.* 1997; Morton *et al.* 2001; Morton *et al.* 2004). Studies investigating potential therapeutic effects of 11βHSD1 inhibition have demonstrated improved insulin sensitivity in animal models of obesity and insulin resistance (Alberts *et al.* 2002; Alberts *et al.* 2003). A recent study using a selective 11βHSD1 inhibitor to treat type 2 diabetes found treatment resulted in significantly reduced fasting blood glucose, as well as improvements in measures of insulin resistance (Rosenstock *et al.* 2010).

The mechanism of tissue-specific dysregulation of 11βHSD1 in obesity remains uncertain, however, given the roles that both inflammation and 11βHSD1 have in the underlying pathogenesis of obesity-linked insulin resistance, it appears that the two may be linked. Indeed, *in vitro* studies have demonstrated that pro-inflammatory cytokines including TNFα and IL-6 up-regulate 11βHSD1 in several cell types including in human pre-adipocytes (Tomlinson *et al.* 2001; Tomlinson *et al.* 2004). These observations raise the possibility that increased intra-adipose 11βHSD1 expression is a manifestation of the pro-inflammatory state in obesity. This chapter will investigate whether inflammation is a regulator of adipose glucocorticoid metabolism.

Hypothesis

The hypothesis of this chapter is that a salicylate-induced improvement in insulin sensitivity is associated with reduced adipose 11βHSD1 expression and activity.

Aims

The aims of this chapter were to investigate:

1) Whether salicylates improve insulin sensitivity in diet-induced obesity.

2) Whether improved insulin sensitivity is associated with reduced adipose 11βHSD1 expression and activity.

3.2 Materials and methods

3.2.1 Investigation of salicylate effects on glucocorticoid metabolism *in vivo*

3.2.1.1 Experimental outline

Mice were continuously infused with sodium salicylate (120mg/kg/day) or vehicle (distilled water) via subcutaneously implanted osmotic mini-pumps for 4 weeks. Mice were weighed twice weekly throughout the course of the experiment. Glucose tolerance tests were carried out on Day 23 or 24. On Day 27 or 28, mice were culled with blood and tissues collected.

3.2.1.2 Animal Maintenance

Adult male C57Bl6 mice were obtained (Harlan, Olac, UK) at 12 weeks of age. Mice were maintained as described (2.5.1). Upon arrival, they were allowed to acclimatise to the new environment for at least one week before any experiments were initiated. Two cohorts of mice were studied:

- A 'lean' cohort of C57Bl/6 mice was maintained on standard chow diet (RMI 801002; Special Diet Services, Witham, UK) over the course of the experiment.
- A diet-induced obese ('DIO') cohort of C57Bl/6 mice was generated by ten weeks of feeding with high fat diet (58% fat, 12% sucrose; D12331, Research Diets, NJ, USA). The mice were maintained on this diet thereafter until the experiment was complete.

3.2.1.3 Mini-pump implantation

To ensure a dose of 120mg/kg/day was administered, two different concentrations of sodium salicylate were made up dependent upon the weight of the mice. For 'lean'

mice, sodium salicylate (1.16g) was dissolved in distilled water (2ml). For obese ('DIO') mice, sodium salicylate (1.52g) was dissolved in distilled water (2ml). Vehicle groups received distilled water. Osmotic mini-pumps were loaded as described (2.5.2.1). Mice were operated on Day 0 using the surgical procedures described (2.5.2.2).

3.2.1.4 Glucose tolerance test

Glucose tolerance tests were carried out as described (2.5.4) on Day 23 or 24.

3.2.1.5 Terminal procedures

Animals were culled on Day 27 or 28 as described (2.5.5).

3.2.1.6 Quantification of mRNA expression

Total RNAs were isolated from snap frozen livers (2.6.1.1), subcutaneous adipose, mesenteric adipose and omental adipose (2.6.1.2) before RNA quantification (2.6.1.4). First strand cDNA was synthesised by RT-PCR (2.6.1.6). The mRNA abundances of 11βHSD1, angiotensinogen (AGT), adiponectin (AdiQ), tumor necrosis factor-α (TNFα), hormone sensitive lipase (HSL), adipose triglyceride lipase (ATGL) and lipoprotein lipase (LPL) were quantified by real-time PCR (2.6.1.8) and normalised against the mean of the transcript levels of a combination of house-keeping genes as outlined in Table 3.1. These combinations of house-keeping genes were selected due to lack of change in expression following treatment.

Tissue	Gene Combination in Lean Mice	P value	Gene Combination in DIO Mice	P value
Liver	TBP + Cyc	*0.14*	18S + TBP	*0.79*
Omental adipose	18S + TBP + Cyc	*0.28*	18S + Cyc	*0.22*
Mesenteric adipose	18S	*0.35*	18S + Cyc	*0.69*
Subcutaneous adipose	18S + TBP	*0.14*	18S + TBP	*0.35*

Table 3.1 Housekeeping genes used for each tissue for real-time PCR. DIO = diet-induced obese; 18S = 18S ribosomal RNA; TBP = TATA-binding protein; Cyc = cyclophilin A. P value indicates results of student's t-test between Vehicle- and Salicylate-treated mice for specific combination of house-keeping genes.

3.2.1.7 Enzymology

11βHSD1 reductase activity was measured in mesenteric and subcutaneous adipose as described (2.6.2.4). In mesenteric adipose, samples from 'lean' mice were incubated with protein concentration (100μg/ml) and substrate concentration (2μM) for 16 hours. Obese 'DIO' samples were incubated with protein concentration (300μg/ml) and substrate concentration (10μM) for 24 hours. In subcutaneous adipose, paucity of tissue in 'lean' mice prevented assessment of activity in these animals. In obese 'DIO' mice, samples were incubated with protein concentration (100μg/ml) and substrate concentration (2μM) for 22 hours.

3.2.1.8 Quantification of glucose in plasma

Glucose in plasma collected during the glucose tolerance test was quantified by hexokinase assay as described (2.7.2).

3.2.1.9 Quantification of insulin in plasma

Insulin in plasma collected during the glucose tolerance test was quantified by ELISA as described (2.7.1).

3.2.1.10 Quantification of triglycerides in plasma

Fasting plasma triglyceride levels from '0' time point during glucose tolerance test were quantified as described (see 2.7.4).

3.2.1.11 Quantification of NEFAs in plasma

NEFAs in plasma collected during the first two time points of the glucose tolerance test ('0' and '15' min) were quantified as described in (2.7.3).

3.2.1.12 Quantification of β-hydroxybutyrate in plasma

Fasting plasma β-hydroxybutyrate levels from '0' time point during glucose tolerance test were quantified as described (see 2.7.6).

3.2.2 Statistics

Data are presented as mean ± SEM and were analysed by unpaired student's t-tests, One-way Analysis of Variance (ANOVA) with Tukey post-hoc tests, or Two-way ANOVA followed by Bonferroni post-hoc tests as appropriate.

3.3 Results

3.3.1 Salicylate effects on 11βHSD1 in lean and diet-induced obese (DIO) mice

3.3.1.1 Body and tissue weights

In both lean C57Bl/6 and DIO mice, salicylate did not induce changes in body weight compared to vehicle treatment (Table 3.2). In lean mice, salicylate did not significantly alter tissue weights, however, there were trends towards increased subcutaneous (S.C.) adipose (*P=0.06*) and decreased liver weight (*P=0.07*). In DIO mice, there was an increase in the ratio of S.C. to omental adipose depots weights with salicylate, as well as a trend for increased S.C. adipose (*P=0.09*).

3.3.1.2 Glucose tolerance test

To investigate the effects of salicylate on insulin sensitivity, plasma glucose and insulin levels were quantified during a glucose tolerance test (Figure 3.1). In lean mice, salicylate increased insulin levels (Figure 3.1b and d), but did not alter glucose levels (Figure 3.1a and c). Plasma glucose levels in DIO mice were significantly elevated compared to lean mice, an effect that was attenuated by salicylate treatment (3.1a and c). Insulin levels in DIO mice, whilst significantly higher than in lean mice, were unaltered by salicylate treatment (Figure 3.1b and d).

Body Weights						
	Lean			DIO		
	Vehicle	Salicylate	p Value	Vehicle	Salicylate	p Value
Weight at start (g)	28.8 ± 0.6	29.2 ± 0.5	*0.67*	38.0 ± 1.2	39.4 ± 1.6	*0.51*
Weight at end (g)	29.2 ± 0.8	29.5 ± 0.7	*0.8*	39.0 ± 1.4	40.3 ± 2.3	*0.65*
Tissue Weights						
	Lean			DIO		
Tissue	Weight as % Body Weight		p Value	Weight as % Body Weight		p Value
	Vehicle	Salicylate		Vehicle	Salicylate	
Liver	4.38 ± 0.13	4.05 ± 0.10	*0.07*	3.51 ± 0.08	3.65 ± 0.10	*0.29*
S.C. Adipose	0.79 ± 0.05	0.94 ± 0.06	*0.06*	0.96 ± 0.12	1.29 ± 0.14	*0.09*
Omental Adipose	0.04 ± 0.01	0.04 ± 0.01	*0.92*	0.15 ± 0.03	0.12 ± 0.02	*0.34*
Mesenteric Adipose	0.69 ± 0.09	0.73 ± 0.10	*0.83*	1.36 ± 0.15	1.589 ± 0.25	*0.44*
Retroperitoneal Adipose	0.43 ± 0.05	0.47 ± 0.06	*0.6*	0.81 ± 0.07	0.92 ± 0.08	*0.28*
Epididymal Adipose	1.52 ± 0.14	1.79 ± 0.15	*0.21*	2.62 ± 0.22	2.74 ± 0.13	*0.65*
Quadricep Muscle	0.50 ± 0.05	0.49 ± 0.04	*0.97*	0.31 ± 0.03	0.28 ± 0.03	*0.50*
Adrenal	0.02 ± 0.001	0.02 ± 0.001	*0.86*	0.01 ± 0.001	0.01 ± 0.001	*0.34*
Ratio S.C:Omental Adipose	25.7 ± 4.8	27.7 ± 1.8	*0.71*	7.6 ± 0.9	11.3 ± 1.1	***0.02***

Table 3.2 Body and tissue weights of lean and diet-induced obese (DIO) mice following salicylate or vehicle treatment for 4 weeks. No significant differences in body weight were observed between vehicle and salicylate treated groups prior to or after treatment. In lean mice, no significant differences were observed in tissue weights (expressed as percentage of body weight) following salicylate treatment. In DIO mice, there was a significant increase in the ratio of S.C. adipose to omental adipose following salicylate treatment. Data are mean ± SEM for n=7-8 per group. Comparisons between Vehicle and Salicylate were by Student's t-test.

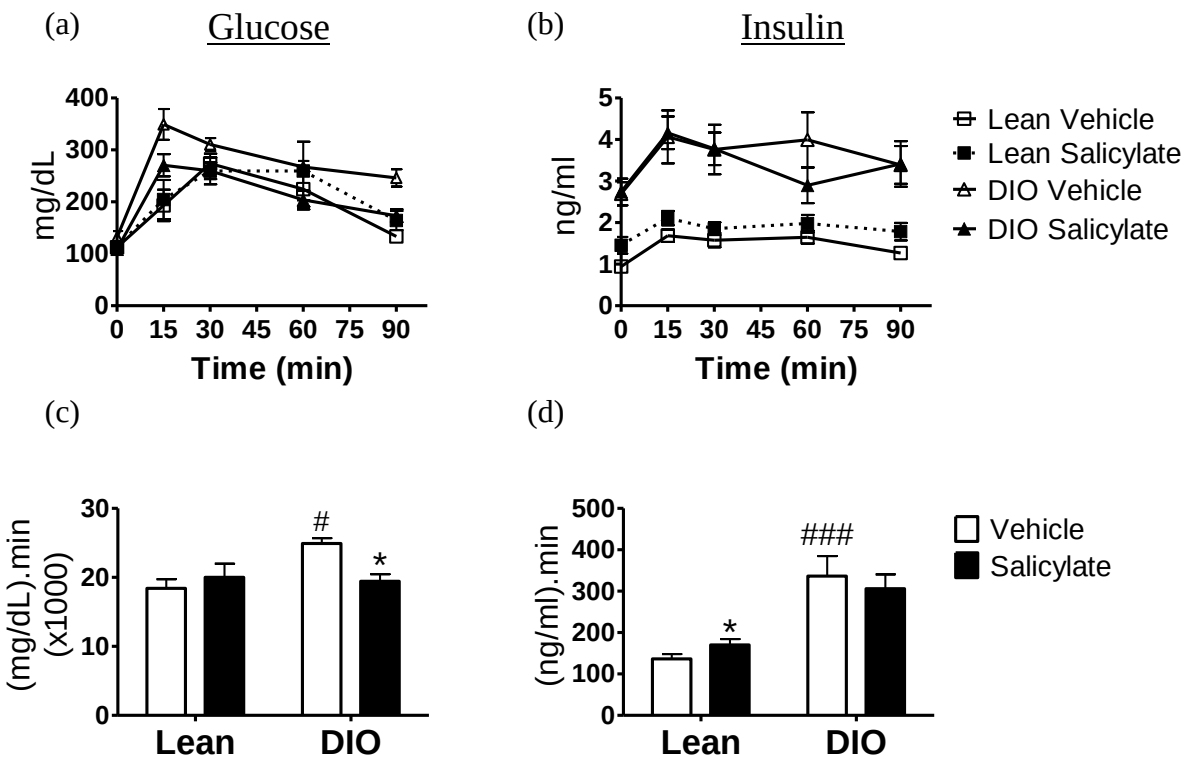

Figure 3.1 Salicylate improved glucose tolerance in DIO mice. Plasma glucose (a) and insulin (b) levels over the course of a glucose tolerance test, with areas under the curve (AUC) shown underneath (c and d respectively), were measured spectrophotometrically. Squares denote lean mice, whilst triangles denote DIO mice. Open symbols/bars denote vehicle treatment, whilst closed symbols/bars denote salicylate treatment. In lean mice, salicylate had no effect on plasma glucose (c), but significantly increased plasma insulin levels (d). Compared to lean mice, diet-induced obese (DIO) had significantly elevated plasma insulin (d), and glucose levels (c) over the course of the test. Salicylate treatment in DIO mice reduced plasma glucose levels (c). Data are mean ± SEM for n=7-8 per group for individual time points and for area under the curve. Comparisons for AUC were by two-way ANOVA with Bonferroni post-hoc tests: *$P<0.05$ vs Vehicle on same diet; $^{\#}P<0.05$, $^{\#\#\#}P<0.001$ vs Lean Vehicle.

3.3.1.3 Plasma NEFA levels

In lean mice, salicylate did not alter fasting plasma NEFA levels or their suppression 15 min after glucose infusion (Figure 3.2). DIO mice had significantly elevated fasting plasma NEFA levels compared with lean mice (Figure 3.2a) and a greatly reduced ability to suppress NEFA release following a glucose bolus (Figure 3.2b). In this obese setting, salicylate reduced fasting plasma NEFA levels (Figure 3.2a), as well as increasing NEFA suppression following a glucose bolus (Figure 3.2b).

3.3.1.4 Plasma triglyceride levels

In lean mice, salicylate treatment did not alter fasting plasma triglyceride levels (Figure 3.3). Compared to lean, DIO mice had significantly elevated fasting triglyceride levels and there was a trend towards a reduction in triglyceride levels (*P=0.06*) with salicylate treatment.

3.3.1.5 Plasma β-hydroxybutyrate levels

Salicylate did not alter fasting β-HB levels in lean mice (Figure 3.4). DIO mice had significantly elevated fasting β-HB levels compared to lean mice, with salicylate significantly reducing these levels.

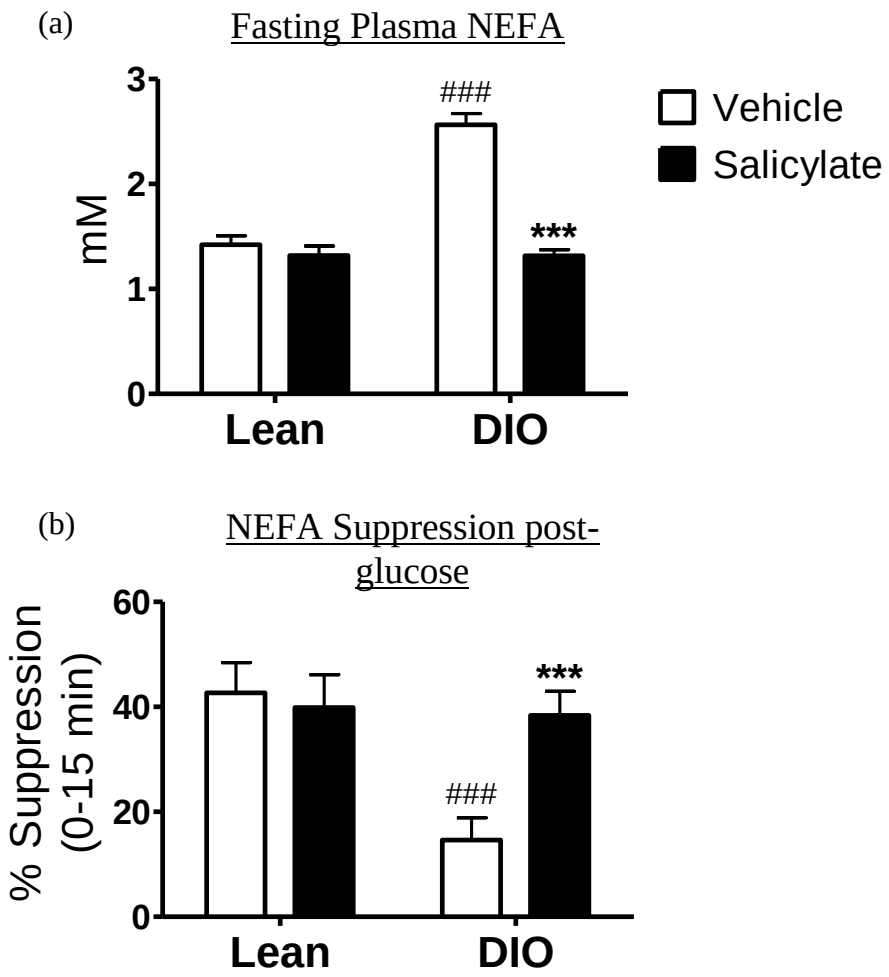

Figure 3.2 Salicylate altered NEFA levels in DIO mice. Fasting plasma non-esterified fatty acids (NEFA) levels (a) and 15 min post-glucose challenge NEFA suppression (b) in lean and diet-induced obese (DIO) mice were measured spectrophotometrically. Open bars denote vehicle treatment, whilst closed bars denote salicylate treatment. In lean mice, salicylate had no effect on fasting NEFA levels (a) or on post-glucose NEFA suppression (b). Compared to lean mice, DIO mice had significantly elevated fasting NEFA levels (a), as well as significantly reduced post-prandial NEFA suppression (b). In DIO mice, salicylate significantly reduced fasting NEFA levels (a) and improved post-prandial NEFA suppression (b). Data are mean ± SEM for n=7-8 per group. Comparisons were by two-way ANOVA with Bonferroni post-hoc tests: ***P<0.001 vs Vehicle on same diet; ### P<0.001 vs Lean Vehicle.

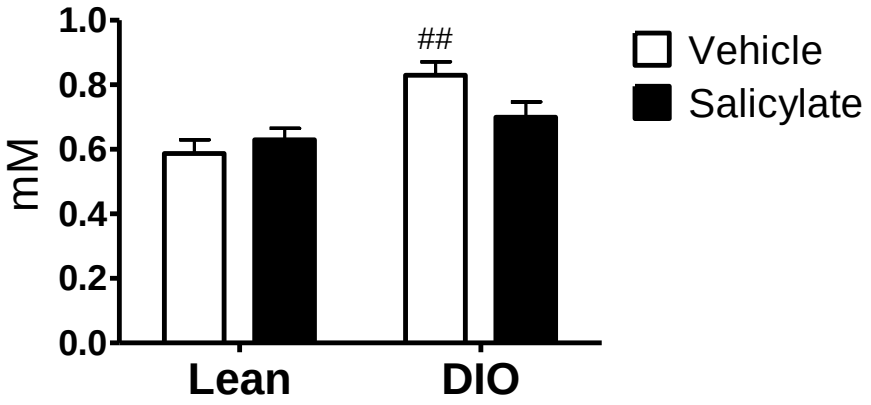

Figure 3.3 Salicylate did not alter triglyceride levels. Fasting plasma triglyceride levels in lean and diet-induced obese (DIO) mice were measured spectrophotometrically. Open bars denote vehicle treatment, whilst closed bars denote salicylate treatment. Salicylate did not affect fasting triglyceride levels in lean mice. DIO mice had significantly elevated triglyceride levels compared to lean mice. Whilst salicylate had no significant effect on triglyceride levels in DIO mice, there was a trend towards a reduction following salicylate treatment (*P=0.08*). Data are mean ± SEM for n=7-8 per group. Comparisons were by two-way ANOVA with Bonferroni post-hoc tests: $^{\#\#}P<0.01$ vs Lean Vehicle.

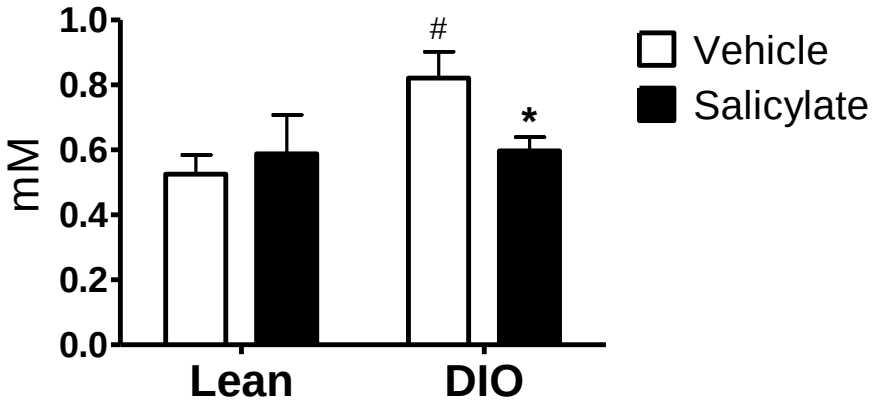

Figure 3.4 Salicylate reduced β-hydroxybutyrate (β-HB) levels in DIO mice. Fasting plasma β-HB levels in lean and diet-induced obese (DIO) mice were measured spectrophotometrically. Open bars denote vehicle treatment, whilst closed bars denote salicylate treatment. In lean mice, salicylate had no effect on fasting β-HB levels. Compared to lean, DIO mice had significantly elevated β-HB levels, with salicylate treatment normalising the levels. Data are mean ± SEM for n=7-8 per group. Comparisons were by two-way ANOVA with Bonferroni post-hoc tests: *P<0.05 vs Vehicle on same diet; # P<0.05 vs Lean Vehicle.

3.3.1.6 11βHSD1 expression and activity

To investigate whether salicylate reduced abundance of 11βHSD1 mRNA, transcript levels were analysed in liver and several adipose depots (Figure 3.5). In lean mice (Figure 3.5a), salicylate did not significantly alter mRNA levels of 11βHSD1 in liver, S.C. adipose or mesenteric adipose, however, there was a trend towards reduced expression in omental adipose (*P=0.08*). In DIO mice (Figure 3.5b), salicylate significantly reduced mRNA levels of 11βHSD1 in omental adipose, with an accompanying trend towards a reduction in mesenteric adipose (*P=0.06*).

The activity of 11βHSD1 was further assessed in adipose tissue to confirm if changes in transcript levels were matched with changes in protein (Figure 3.6). Paucity of omental adipose tissue prevented assessment of enzyme activity in this depot, and so mesenteric adipose was assessed instead (Figure 3.6a). In both lean and DIO mice, salicylate treatment significantly reduced 11βHSD1 activity in mesenteric adipose. The necessity for a much greater substrate concentration in DIO mice (10μM vs 2μM) in order to detect equivalent turnover of substrate, due to suppression of 11βHSD1, meant a direct comparison of conversion rates was not possible. However, extrapolation using the Michaelis-Menten equation and previously reported Vmax and Km values for the enzyme (Mitic 2009) allowed a comparison of reaction velocity in both groups, revealing that 11βHSD1 enzyme activity in the mesenteric adipose of DIO mice was significantly downregulated compared to lean mice (Figure 3.6a). In S.C. adipose (Figure 3.6b), shortage of tissue in lean mice prevented assessment of enzyme activity in these animals. However, in DIO mice S.C. adipose 11βHSD1 enzyme activity was not significantly altered following salicylate treatment.

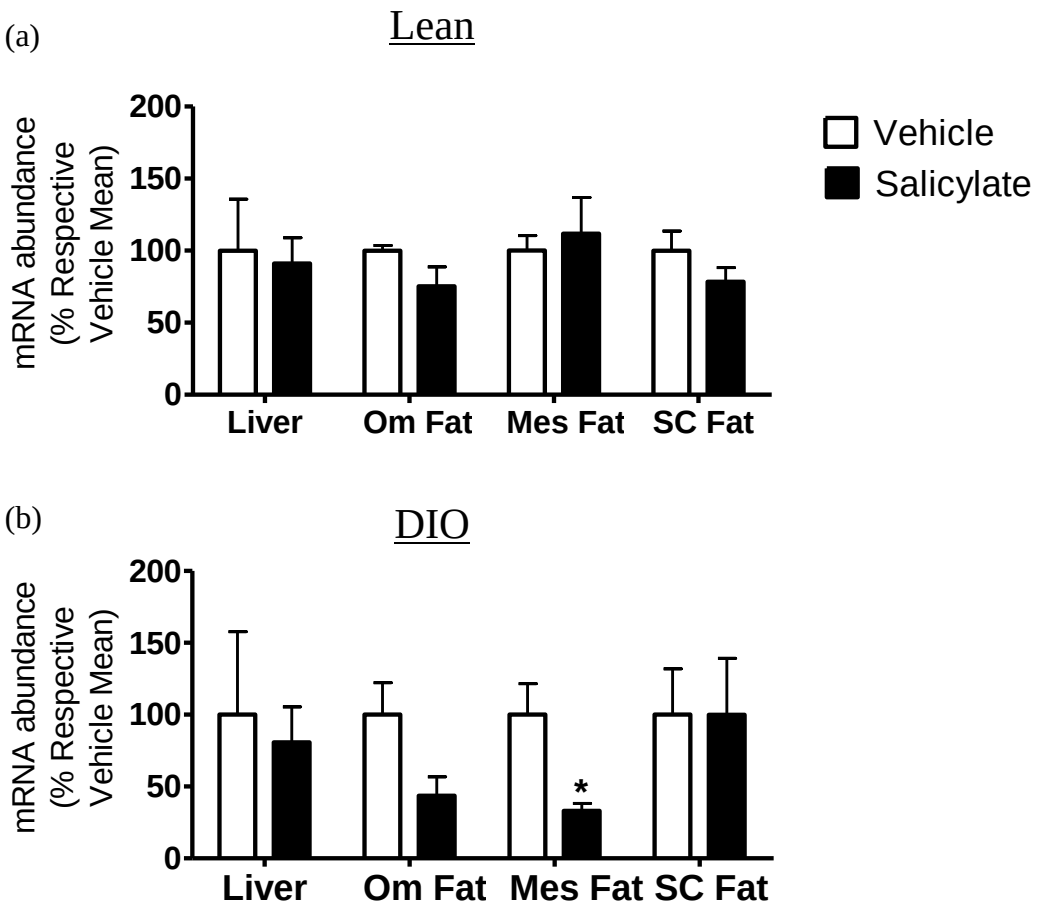

Figure 3.5 Salicylate reduced 11βHSD1 transcript levels in omental adipose. mRNA levels of 11βHSD1 in liver, omental adipose (Om Fat), mesenteric adipose (Mes Fat) and subcutaneous adipose (SC Fat) were determined by real-time PCR in lean (a) and diet-induced obese (DIO) (b). In lean mice (a), salicylate treatment (black bars) had no significant effect on 11βHSD1 transcript levels, however there was a trend towards reduced expression in omental adipose (*P=0.08*). In DIO mice (b), salicylate significantly reduced 11βHSD1 expression in omental adipose compared to vehicle (open bars), with an accompanying trend in mesenteric adipose (*P=0.06*). Data are mean ± SEM, expressed as % of the respective Vehicle mean. N=6-8 per group. Comparisons were by Student's unpaired t-test: *P<0.05 vs Vehicle on same diet.

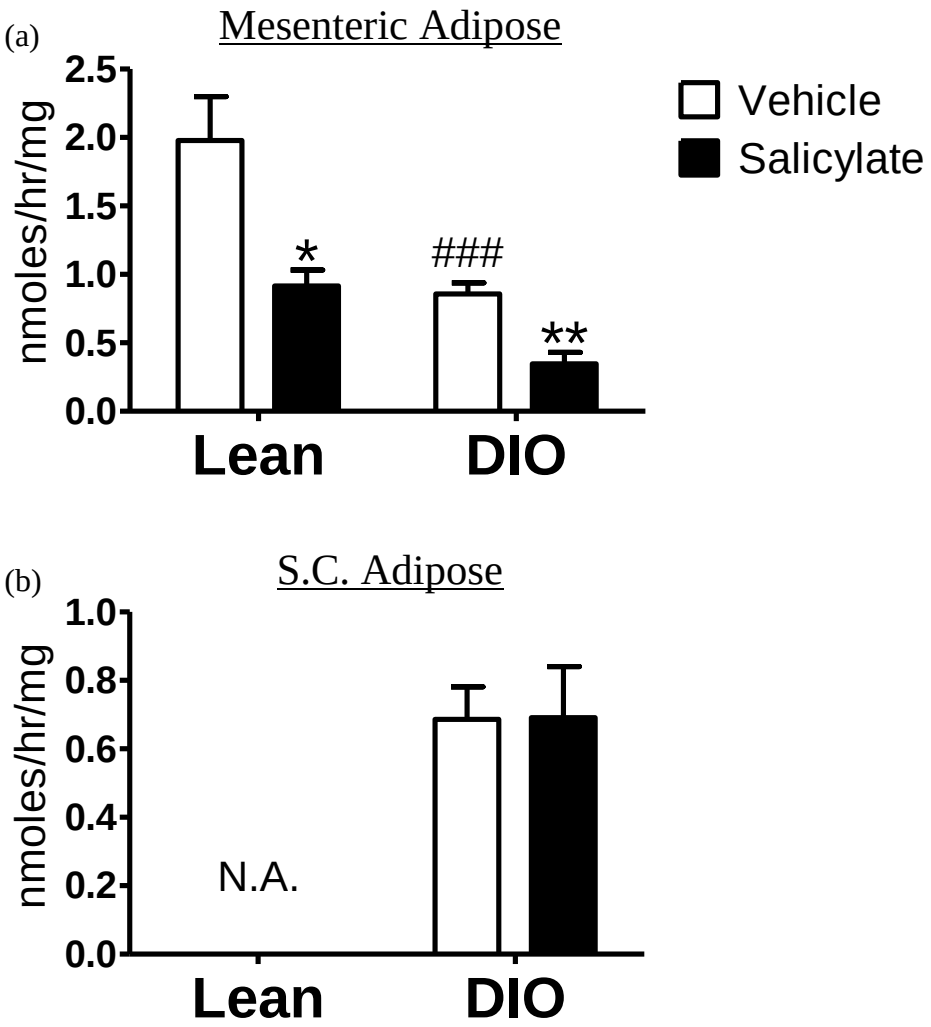

Figure 3.6 Salicylate reduced mesenteric adipose 11βHSD1 activity. Enzyme velocity was measured in mesenteric adipose (a) and subcutaneous (S.C) adipose (b). In mesenteric adipose, salicylate significantly reduced 11βHSD1 activity in both lean and diet-induced obese (DIO) mice. DIO mice had significantly reduced activity in mesenteric adipose compared to lean mice (a). In S.C. adipose (b), salicylate had no effect in DIO mice. Insufficient tissue in lean mice prevented measurement of activity in S.C. adipose of these animals. Data are mean ± SEM for n=7-8 per group. Comparisons were by two-way ANOVA with Bonferroni post-hoc tests: *P<0.05, **P<0.01 vs Vehicle on same diet; ###P<0.001 vs Lean Vehicle. N.A. = not assessed.

3.3.1.7 Adipokine expression in adipose tissue

In lean mice, salicylate had limited effects on adipokine expression in adipose tissue. In omental adipose (Figure 3.7a), salicylate significantly reduced TNFα mRNA levels, but had no effect on adiponectin (AdiQ), lipoprotein lipase (LPL), adipose triglyceride lipase (ATGL) or hormone sensitive lipase (HSL) mRNAs. In mesenteric adipose (Figure 3.8a), salicylate significantly increased adiponectin mRNA levels, but had no effect on any other transcripts. In S.C. adipose (Figure 3.9a), salicylate significantly increased mRNA levels of TNFα, with a trend towards reduced AdiQ (*P=0.06*). In DIO mice, salicylate had much more pronounced effects on adipokine transcript expression, predominantly within the visceral adipose depots including omental and mesenteric. In omental adipose (Figure 3.7b), salicylate significantly reduced mRNA levels of AGT, TNFα, LPL and ATGL, whilst increasing AdiQ. In mesenteric adipose (Figure 3.8b), a similar pattern resulted from salicylate treatment. In addition to AGT and ATGL transcript levels being significantly reduced by salicylate, there were trends towards reduced TNFα (*P=0.06*), as well as increased AdiQ (*P=0.06*), whilst LPL, in contrast to omental adipose, appeared to be increased although not significantly (*P=0.07*). In S.C. adipose (Figure 3.9a), salicylate significantly increased both AdiQ and LPL mRNA levels, but had no effect on the transcript levels of other adipokines.

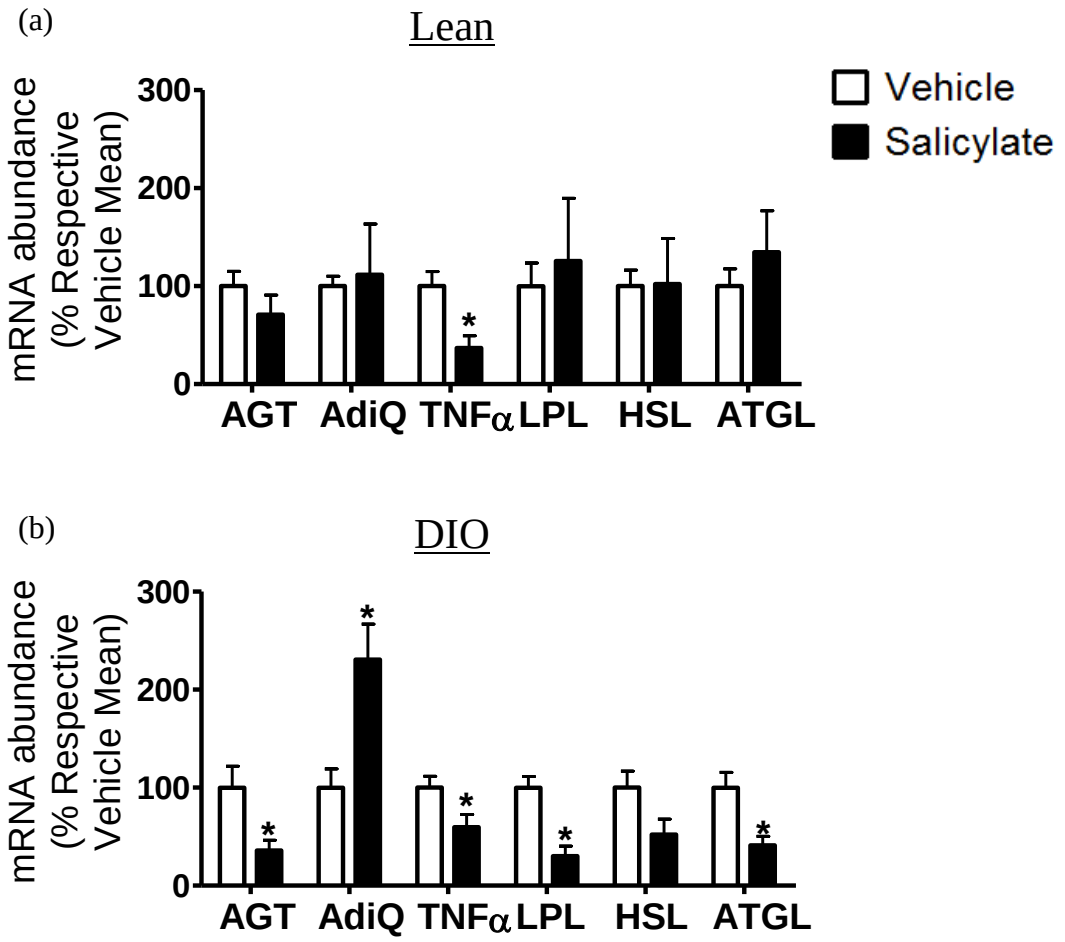

Figure 3.7 Salicylate altered omental adipose transcript levels. mRNA levels of angiotensinogen (AGT), adiponectin (AdiQ), tumor necrosis factor-α (TNFα), lipoprotein lipase (LPL), hormone sensitive lipase (HSL) and adipose triglyceride lipase (ATGL) were determined by real-time PCR in lean (a) and diet-induced obese (DIO) (b) mice. In lean mice (a), salicylate significantly reduced TNFα mRNA levels. In diet-induced obese mice (b), salicylate significantly reduced TNFα, LPL and ATGL, whilst increasing adiponectin mRNA levels. Data are mean ± SEM, expressed as % of the respective Vehicle mean. N=6-8 per group. Comparisons were by Student's unpaired t-test: **P*<0.05 vs Vehicle.

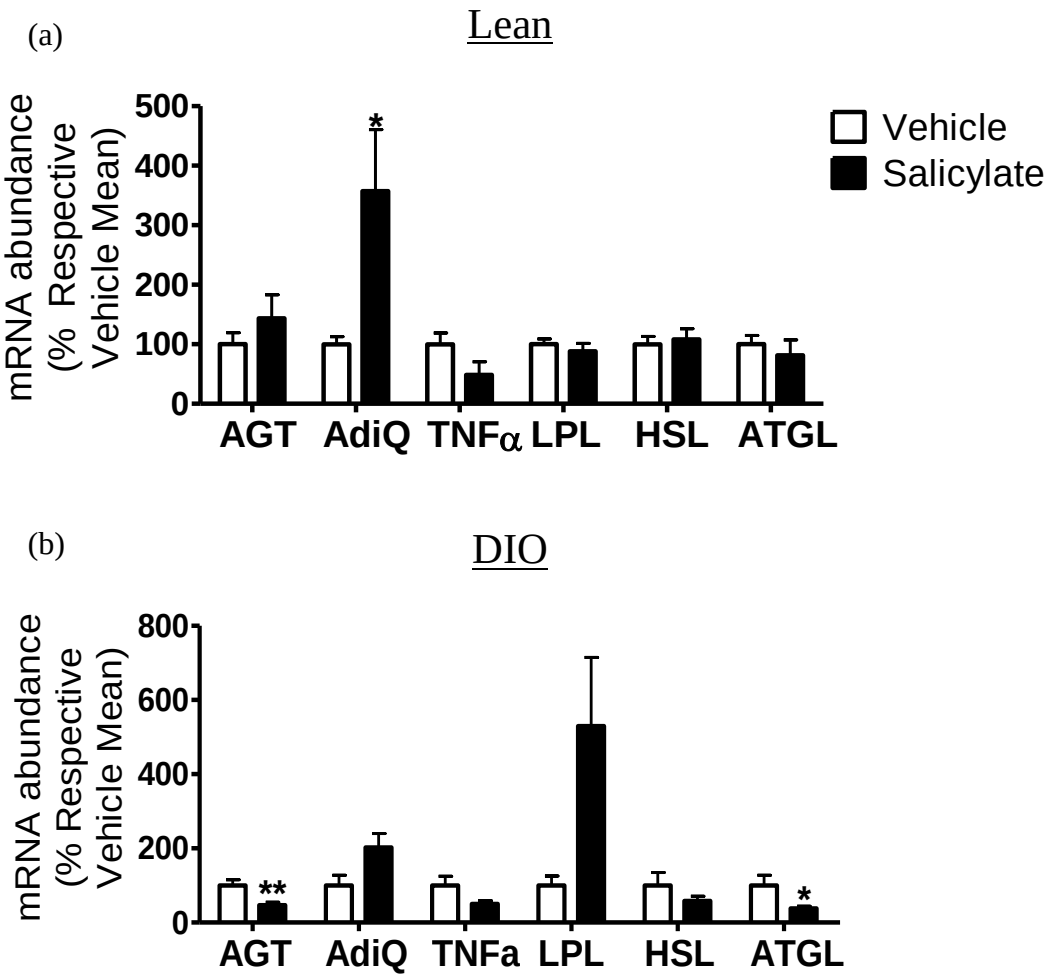

Figure 3.8 Salicylate alters mesenteric adipose transcript levels. mRNA levels of angiotensinogen (AGT), adiponectin (AdiQ), tumor necrosis factor-α (TNFα), lipoprotein lipase (LPL), hormone sensitive lipase (HSL) and adipose triglyceride lipase (ATGL) were determined by real-time PCR in lean (a) and diet-induced obese (DIO) (b) mice. In lean mice (a), salicylate significantly increased adiponectin expression. In DIO mice (b), salicylate significantly reduced ATGL expression, with trends towards reduced TNFα (*P=0.*06) as well as increased adiponectin (*P=0.*06) and LPL (*P=0.*07). Data are mean ± SEM, expressed as % of the respective Vehicle mean. N=6-8 per group. Comparisons were by Student's unpaired t-test: **P*<0.05, ***P*<0.01 vs Vehicle.

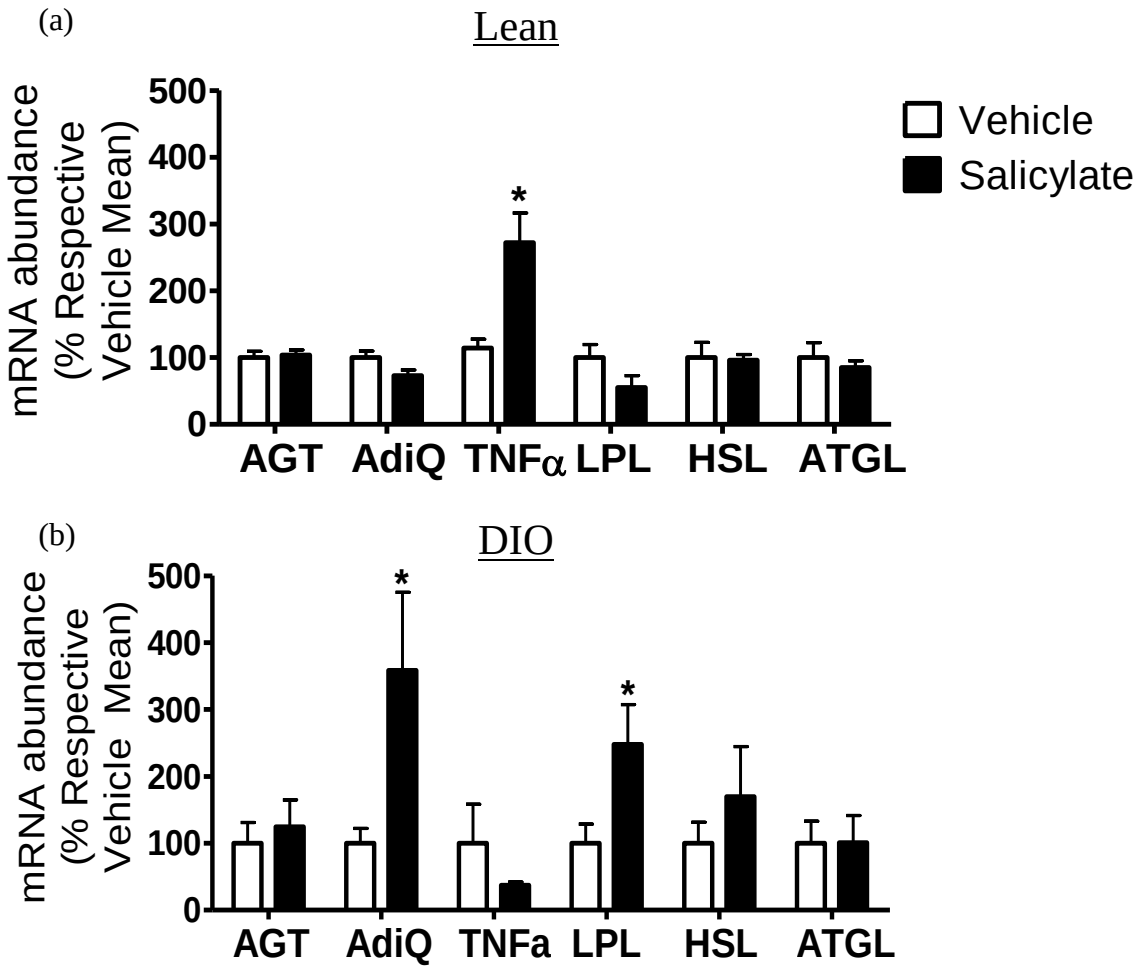

Figure 3.9 Salicylate alters subcutaneous (S.C.) adipose transcript levels. mRNA levels of angiotensinogen (AGT), adiponectin (AdiQ), tumor necrosis factor-α (TNFα), lipoprotein lipase (LPL), hormone sensitive lipase (HSL) and adipose triglyceride lipase (ATGL) were determined by real-time PCR in lean (a) and diet-induced obese (DIO) (b) mice. In lean mice (a), salicylate significantly increased TNFα expression. In DIO mice (b), salicylate significantly increased adiponectin and LPL expression. Data are mean ± SEM, expressed as % of the respective Vehicle mean. N=6-8 per group. Comparisons were by Student's unpaired t-test: **P*<0.05 vs Vehicle.

3.4 Discussion

Whilst recent years have seen tremendous progress in understanding the relationship between inflammation and insulin resistance, many of the underlying mechanisms remain unclear. Initial clues as to the role of inflammation in insulin resistance date back to over a century ago, when it was discovered that high doses of sodium salicylate, an anti-inflammatory drug, were capable of diminishing glycosuria in diabetic patients (Reid *et al.* 1957). Despite this, it was not until recently that the mechanisms behind this hypoglycemic effect were reinvestigated. In this chapter, we have demonstrated that the ability of salicylate to improve insulin sensitivity in diet-induced obese mice is associated with a reduction in 11βHSD1 expression and activity.

Salicylate compounds, including sodium salicylate and acetylsalicylic acid, improve insulin sensitivity in both rodent and human conditions of insulin resistance (Yuan *et al.* 2001; Alberts *et al.* 2003; Goldfine *et al.* 2010; Rosenstock *et al.* 2010). Rodent models used to demonstrate this to date include genetically obese *ob/ob* mice (Yuan *et al.* 2001) and lipid infusion in rats (Kim *et al.* 2001). Given that human obesity is predominantly polygenic in origin, C57Bl/6 mice fed a high-fat diet are commonly used as they are considered more representative of the human nature of the condition, although this model has, to our knowledge, not previously been used to investigate the potential of salicylates to improve insulin sensitivity. It is suggested that inhibition of inflammatory pathways, specifically via IKKβ and NFκB, are crucial in salicylate action (Yuan *et al.* 2001). To investigate the mechanism behind the possible insulin sensitising effects of salicylates, mice were infused over a 4 week period with a high dose of sodium salicylate (120mg/kg/day), with insulin sensitivity being measured though a glucose tolerance test (GTT). This dose and treatment period was chosen as it has previously been shown to be effective in improving glucose tolerance and insulin sensitivity in rodent models of obesity (Yuan *et al.* 2001).

Initial work in lean mice revealed that, contrary to improving insulin sensitivity, salicylate induced mild insulin resistance, evidenced by an increase in insulin levels without a change in glucose levels over the course of the GTT. Whilst this was unexpected, it is perhaps not entirely unsurprising. Despite numerous reports of salicylate-induced improvements in insulin sensitivity, the metabolic effects of salicylates still remain somewhat contentious. There are several reports describing an increase in insulin concentration following salicylate treatment, due to both increased secretion and impaired clearance (Newman and Brodows 1983; Bratusch-Marrain *et al.* 1985). This is not associated with hypoglycaemia, suggesting that salicylates can induce insulin resistance. A possible mechanism by which salicylates may induce insulin resistance is through inhibition of prostaglandin production. Prostaglandins function as a negative feedback mechanism, inhibiting TNFα production, and so their inhibition could increase TNFα levels (Netea *et al.* 2001). However, what appears more than likely is that a lack of underlying insulin resistance plays a role in the inability of salicylate to improve insulin sensitivity. In healthy men, lipid infusion induces an insulin resistant state that is improved with acetylsalicylic acid treatment (Mohlig *et al.* 2006). However, acetylsalicylic acid treatment in the absence of lipid-induced insulin resistance has no beneficial effect (Mohlig *et al.* 2006). A similar study in rats again found that treatment with salicylate ameliorated insulin resistance induced by lipid infusion, yet salicylate treatment alone had no effect (Park *et al.* 2007). Furthermore, this study found that salicylate treatment reversed the decrease in anti-inflammatory markers in muscle and the inhibitory serine phosphorylation of IRS-1 in the liver induced by lipid infusion. However, salicylate treatment alone had no effect on these parameters. This evidence supports what was found in our study, that in the lean state, salicylate treatment does not improve insulin sensitivity. This presents a similar scenario to the use of PPARγ agonists, such as thiazolidinediones, which only have beneficial effects in improving insulin sensitivity in the obese state (Ribon *et al.* 1998).

To investigate whether obesity-induced insulin resistance was necessary in order to observe beneficial effects of salicylate, mice were fed a high-fat diet for 10 weeks

prior to initiation of salicylate treatment. Compared to lean mice, these diet-induced obese (DIO) mice exhibited a phenotype typical of the metabolic syndrome, with hyperinsulinemia, hyperglycemia and dyslipidemia. In contrast to their lean counterparts, DIO mice responded favourably to salicylate, with reduced plasma glucose levels following treatment, indicating improved glucose tolerance and insulin sensitivity. Although there were no statistically significant differences in individual tissue weights seen following treatment, there was an increased ratio of S.C. to omental adipose, indicating that salicylate may cause a subtle redistribution of body fat, causing these mice to store fat preferentially in S.C. depots.

One of the main aims of this chapter was to investigate whether the beneficial effects of salicylate were associated with any changes in adipose expression of the glucocorticoid-regenerating enzyme 11βHSD1. In lean mice, no significant changes were seen in mRNA levels of 11βHSD1 in various adipose depots or indeed the liver, although there was a trend towards reduced expression in omental adipose. A lack of tissue meant that 11βHSD1 activity could not be analysed in omental adipose, and so this was instead assessed in a similar central depot, mesenteric adipose, and found to be significantly reduced. Despite this depot-specific downregulation of 11βHSD1, no improvements were seen in insulin sensitivity. However, this may simply be due to the fact that these were healthy, lean animals, with no further benefits to be gained from reducing 11βHSD1 levels. In contrast to lean mice, an improvement in insulin sensitivity in DIO mice following salicylate treatment was associated with a downregulation of 11βHSD1 mRNA levels in both omental and mesenteric adipose depots. Enzyme activity was also significantly reduced in mesenteric adipose following salicylate treatment. This salicylate-induced downregulation of 11βHSD1 demonstrates an *in vivo* link between inflammation and 11βHSD1 levels within adipose tissue, consistent with the theory that inflammatory mediators may regulate the expression of this enzyme. However, the lack of salicylate-induced changes in 11βHSD1 mRNA expression or enzyme activity in peripheral, S.C. adipose, indicates that salicylate effects on 11βHSD1 appear localised to visceral adipose depots.

In order to understand the reason behind these depot-specific effects, it is important to consider the heterogeneous nature of adipose tissue. Visceral adipose tissue is known to be more metabolically important than peripheral depots due to its strong association with insulin resistance, type 2 diabetes and cardiovascular disease (Wajchenberg 2000; Mathieu *et al.* 2010). Even in those who are not overweight, a centralised distribution of adiposity leads to increased risk of metabolic disease. Depot-specific differences between omental and S.C. adipose have been documented for triglyceride uptake, lipolysis, adipocyte size and number, as well as density of GR (Wajchenberg 2000). Furthermore, the inflammatory state of these various adipose depots has been shown to be distinct. Accumulating evidence suggests that visceral adipose is a major source of inflammatory molecules (Fain 2010). In obese individuals with higher visceral adiposity, there is greater infiltration of macrophages into visceral adipose than subcutaneous adipose, enhancing production of pro-inflammatory cytokines in this depot (Cancello *et al.* 2006; Lee *et al.* 2009). Additionally, previous work has shown that pro-inflammatory cytokines are capable of increasing 11βHSD1 expression in both omental and S.C. adipose stromal cells (Tomlinson *et al.* 2001). Given the greater levels of inflammation in obese visceral adipose tissue, it may be that salicylates exert a greater influence on 11βHSD1 expression and activity due to their improved anti-inflammatory properties in visceral adipose. Indeed, in our study, mRNA levels of the pro-inflammatory cytokine TNFα were significantly reduced in omental adipose, with a strong trend towards an accompanying reduction in mesenteric adipose as well, supporting the anti-inflammatory efficacy of salicylate. However, changes in mRNA profiles of inflammatory mediators were not seen in S.C. adipose. Whether this lack of effect in S.C. adipose is attributable to a lack of a direct effect of salicylate upon inflammatory cytokines or a consequence of a lack of downregulation of 11βHSD1 remains to be elucidated.

In terms of changes in the transcript levels of other genes in the various adipose depots, one can only speculate as to whether these are a result of reducing inflammation or a result of reduced glucocorticoid action. Certainly, glucocorticoids

are capable of directly influencing expression of these molecules, and given the greater density of GR in visceral adipose (Bjorntorp 1995), any changes in 11βHSD1 expression or activity would likely have more pronounced effects in such central depots. Adiponectin is one of the major proteins released by adipose, with circulating levels principally controlled by production in adipose (Arita *et al.* 1999; Kadowaki *et al.* 2006; Takemura *et al.* 2006). Expression of this adipokine is inversely associated with obesity and insulin resistance, suggesting alteration in adiponectin homeostasis may play a role in the development of insulin resistance. Both glucocorticoids and TNFα have been shown to have effects on adiponectin expression (Fasshauer *et al.* 2002; Fontana *et al.* 2007; Hector *et al.* 2007). *In vitro* studies in murine adipocytes illustrated that dexamethasone reduces adiponectin gene expression (Fasshauer *et al.* 2002). A study investigating effects of both exogenous and endogenous glucocorticoid exposure on adiponectin levels in men revealed that administration of glucocorticoids to healthy humans reduced circulating adiponectin levels (Fallo *et al.* 2004). Furthermore, a comparison between non-obese Cushing's patients and their non-obese controls demonstrated endogenous hypercortisolism reduces adiponectin levels (Fallo *et al.* 2004). In terms of TNFα, studies in murine adipocytes have also shown that this pro-inflammatory mediator suppresses adiponectin expression, whilst the anti-inflammatory properties of adiponectin were also demonstrated as it suppressed release of TNFα, indicating that these molecules negatively regulate each other (Fasshauer *et al.* 2002). In our study, transcript levels of adiponectin in salicylate-treated DIO mice were significantly increased in both omental and S.C. adipose, with a strong trend towards upregulation in mesenteric adipose. Given the decrease in TNFα expression in visceral adipose, it is possible that a reduction in inflammation facilitates the increase in adiponectin. Alternatively, reduced expression and activity of 11βHSD1 in visceral adipose reduces exposure to glucocorticoids, which would normally act to suppress adiponectin levels. Whilst one of these pathways may explain changes in visceral adipose depots, it does not offer an explanation for the increased adiponectin expression in S.C. adipose, given that neither TNFα nor 11βHSD1 expression was altered. However, this rise in S.C. adipose adiponectin may be a result of an improvement in whole body insulin sensitivity.

Perhaps the most striking depot-specific differences in DIO mice were observed in the transcript levels of LPL, with a significant increase in S.C. adipose and a decrease in omental adipose. This enzyme hydrolyses triglycerides, breaking them down into NEFAs and glycerol, thus enabling NEFA uptake by adipose. As such, LPL levels correlate positively with adiposity. In men, the uptake of triglycerides is significantly higher in omental compared with subcutaneous adipose (Marin *et al.* 1992). Further work revealed that, in men, LPL expression and activity were greater in intra-abdominal (visceral) adipocytes than in S.C. adipocytes, perhaps explaining the greater tendency for men to accumulate fat in central, visceral depots (Marin *et al.* 1992). In terms of our study, the differential effects on LPL mRNA expression would seem to support the subtle redistribution of body fat observed in these animals, and may provide an explanation as to why this occurs. Both insulin and glucocorticoids act to increase LPL levels, however the effects of insulin are much less pronounced in omental adipocytes than S.C. adipocytes (Zierath *et al.* 1998; Karpe and Tan 2005). This, coupled with a reduction in glucocorticoid levels through downregulation of 11βHSD1, may explain the reduction in omental LPL transcript levels. However, it should be noted whilst these changes in mRNA levels provide evidence of altered fatty acid metabolism via LPL, it would be more robust to measure activity of this enzyme in order to gain a clearer understanding of the changes taking place.

In terms of adipose depot-specific differences in lipolysis, previous work reports that catecholamine-stimulated lipolysis is much higher in omental compared to S.C. adipose (Wajchenberg 2000; Karpe and Tan 2005). Pro-inflammatory cytokines, including TNFα, have been shown to induce lipolysis through two mechanisms. Firstly, TNFα elevates intracellular levels of second messenger cyclic adenosine monophosphate (cAMP), activating protein kinase A (PKA), which in turn phosphorylates and activates HSL and ATGL (Bezaire and Langin 2009). A second, more indirect mechanism through which TNFα enhances lipolysis is achieved through serine phosphorylation of insulin receptor substrate-1 (IRS-1) (Gao *et al.*

2002; Draznin 2006). Such phosphorylation results in the inhibition of the insulin signalling cascade which would normally act to suppress adipocyte lipolysis. To investigate if salicylate had any effects on adipocyte lipolysis, we analysed transcript levels of HSL and ATGL in several adipose depots. ATGL mRNA levels were significantly reduced in both mesenteric and omental adipose following salicylate treatment. This may be a direct effect of salicylate treatment or an indirect effect via improved insulin sensitisation. Despite this, it has been shown previously that sodium salicylate treatment alters lipolytic activity of these enzymes without altering protein levels. As such, it may be that salicylate treatment in this study also reduced activity of HSL and ATGL. Whilst enzyme activity was not measured here, it would undoubtedly provide a clearer picture of alterations in lipolysis.

With regard to lipolysis, insulin is anti-lipolytic and normally acts as the master regulator of lipolysis, ensuring its suppression after a meal. However, as mentioned above, its effects are much less pronounced in omental adipose tissue, meaning that other hormones are likely to contribute more to regulation of omental adipose lipolysis. Glucocorticoids are known to increases lipolysis in adipose through induction of the two major lipolytic enzymes HSL and ATGL (Macfarlane *et al.* 2008; Xu *et al.* 2009). This is believed to occur through an elevation in intracellular levels of cAMP, which results from glucocorticoid-mediated reduction in PDE3, an enzyme responsible for the breakdown of cAMP. Whilst reduced glucocorticoid levels in these adipose depots has not been demonstrated, the down-regulation of angiotensinogen (AGT) in depots with a concordant down-regulation of 11βHSD1 provides strong support for a reduction in adipose glucocorticoid levels, as AGT contains GREs within its promoter and so is directly affected by the local levels of glucocorticoids (Olson *et al.* 1991).

These changes in the expression levels of enzymes involved in lipolysis may go some way to explaining the salicylate-induced reduction in plasma NEFA levels in DIO mice. The association of circulating NEFA levels with obesity and insulin

resistance has been well documented, with evidence in both rodents and humans indicating that plasma levels are elevated in obesity, with NEFAs being implicated in the pathogenesis of insulin resistance (Zierath *et al.* 1998; Griffin *et al.* 1999; Karpe and Tan 2005). Early studies have demonstrated the ability of salicylates to reduce plasma NEFA levels (Reid *et al.* 1957; Bizzi and Carlson 1965). In our study, DIO mice had significantly elevated fasting NEFA levels compared to lean mice, an increase that was markedly attenuated with salicylate treatment. Whilst suppression of lipolytic enzymes would undoubtedly reduce NEFA release into the circulation, it is difficult to argue that such a reduction is solely the result of changes in lipolysis in visceral adipose. S.C. adipose contributes to the majority of circulating NEFA levels, whilst the contribution of visceral adipose is relatively small (Karpe and Tan 2005). However, the anatomical site of visceral adipose lipolysis is believed to be significantly more important, as released NEFAs directly enter the portal circulation which connects to the liver. To investigate possible changes in liver exposure to NEFAs, we analysed circulating levels of the ketone body β-hydroxybutyrate (β-HB). During the fating state, NEFAs derived from adipose tissue lipolysis serve as a fuel other tissues, notably the liver (Sunny *et al.* 2010). Within hepatocytes, NEFAs are converted to acyl CoA, which is either used for the synthesis of triglycerides in lipoproteins or transported to the mitochondria for oxidation. Each cycle of β-oxidation produces acetyl CoA, which is converted, downstream, to β-HB and released into the circulation. In a period of extended fasting, this ketone body production provides a critical alternative substrate for oxidation by extra-hepatic tissues, in particular the central nervous system, which is normally dependent on glucose metabolism (Sunny *et al.* 2010). If we consider a metabolic state in which circulating NEFA levels are greatly elevated, such as in obesity, we can see how this leads to increased circulating levels of ketone bodies. An increased flux of NEFAs to the liver leads to increased production of acetyl CoA, which exceeds cellular energy requirement. As such, a greater amount of acetyl CoA is shunted towards β-oxidation, leading to increased production and release of β-hydroxybutyrate. Indeed, it has been shown that in mice fed a high-fat diet, fasting hepatic production of ketone bodies was significantly elevated (Sunny *et al.* 2010). Similarly, in our study, we have also shown that obesity increased fasting plasma β-hydroxybutyrate levels,

with DIO mice showing significantly higher levels compared to lean mice. Interestingly, salicylate treatment in DIO mice attenuated this increase. Two possible explanations may exist for this. Firstly, and perhaps more likely, is that the decrease in ketone body production is a direct result of reduced circulating NEFA levels, meaning reduced NEFA flux to the liver. Another possibility is that salicylate directly impacts upon hepatic β-oxidation, reducing expression of genes involved in oxidation and ketone body formation, including acetyl coenzyme A carboxylase (ACC), malonyl coenzyme A decarboxylase (MCD) and mitochondrial 3-hydroxy-3-methylglutarate coenzyme A synthase (mHMG CoA synthase). However, this appears unlikely given the drop in NEFA levels and so was not explored further.

In summary, treatment of DIO mice with anti-inflammatory salicylate improves insulin sensitivity and reduces circulating NEFA levels. Accompanying this improved metabolic profile was a reduction in the expression and activity of 11βHSD1 in central, visceral adipose depots, demonstrating for the first time *in vivo*, that adipose inflammation and glucocorticoid metabolism are linked. However, it remains unclear whether the salicylate-induced downregulation of adipose 11βHSD1 is a result of reduced adipose inflammation, or if salicylates directly reduce 11βHSD1 expression, which in turn regulates the inflammatory state of adipose. Interestingly, recent *in vitro* treatment of murine 3T3-L1 adipocytes with acetylsalicylic acid resulted in a downregulation of 11βHSD1 mRNA levels (C Esteves *et al*., unpublished data). This demonstrates that salicylate suppresses 11βHSD1 expression in the absence of an underlying inflammatory state, potentially indicating that the effects of salicylate on 11βHSD1 are direct, implicating a role for this enzyme in the regulation of adipose inflammation. This will be explored further in the next chapter.

Chapter 4

The role of 11βHSD1 in the insulin sensitising effects of salicylate in obesity

4.1 Introduction

As shown in Chapter 3, the anti-inflammatory drug sodium salicylate is capable of improving insulin sensitivity in diet-induced obese (DIO) mice, a benefit that occurs alongside a down-regulation of both expression and activity of the glucocorticoid-regenerating enzyme 11βHSD1 in visceral adipose, specifically in the omental and mesenteric depots. This raises the possibility that 11βHSD1 is a crucial component in the underlying mechanism through which salicylates improve insulin sensitivity. However, given that pro-inflammatory cytokines increase 11βHSD1 expression *in vitro* in adipocytes (Tomlinson *et al.* 2001), it remains to be seen whether salicylate-induced down-regulation of 11βHSD1 is a result of reduced inflammation, or if the effects are direct. This chapter will investigate whether salicylate has the same insulin sensitising effect in DIO mice lacking 11βHSD1 as it has in normal wild type DIO mice.

Hypothesis

The hypothesis of this chapter is that salicylate will not improve insulin sensitivity in mice lacking the enzyme 11βHSD1.

Aims

The aims of this chapter were to investigate:

1) Whether salicylate improves insulin sensitivity in DIO mice lacking 11βHSD1.

2) Which of the downstream effects of salicylate treatment on insulin sensitivity and metabolism are dependent upon 11βHSD1.

4.2 Materials and methods

4.2.1 Experimental outline

The experimental procedure is as described in section 3.2.1.

4.2.2 Animal Maintenance

Homozygous male 11βHSD1-deficient (HSD1KO) mice were bred from an in-house colony (Kotelevtsev *et al.* 1997) on a C57Bl/6 background (>10 generations back-cross) with corresponding wild type (WT) controls (Harlan Olac, UK). Diet-induced obesity was induced from 12 weeks of age through feeding with high fat diet (58% fat, 12% sucrose; D12331, Research Diets, NJ, USA), until body weight was matched with DIO mice from 3.2.2.2 (approximately 38g). Mice were maintained as described (2.5.1).

4.2.3 Mini-pump implantation

Osmotic mini-pumps were loaded with solutions of sodium salicylate or vehicle (distilled water) (2.5.2.1). Mice were operated on Day 0 using the surgical procedures described (2.5.2.2).

4.2.4 Glucose tolerance test

Glucose tolerance tests were carried out as described (2.5.4) on Day 23 or 24.

4.2.4 Terminal procedures

Animals were culled on Day 27 or 28 as described (2.5.5).

4.2.5 Molecular biology: quantification of mRNA abundance of genes of interest

Total RNAs were isolated from snap frozen livers (2.6.1.1), subcutaneous adipose, mesenteric adipose and omental adipose (2.6.1.2) before RNA quantification (2.6.1.4). First strand cDNA was synthesised by RT-PCR (2.6.1.6). The mRNA abundances of 11βHSD1, angiotensinogen (AGT), adiponectin (AdiQ), tumour necrosis factor-α (TNFα), hormone sensitive lipase (HSL), adipose triglyceride lipase (ATGL) and lipoprotein lipase (LPL) were quantified by real-time PCR (2.6.1.8) and normalised against the mean of the transcript levels of a combination of house-keeping genes as outlined in Table 4.1. These combinations of house-keeping genes were selected due to lack of change in expression following treatment.

4.2.6 Enzymology

11βHSD1 reductase activity was measured in mesenteric and subcutaneous adipose of WT-DIO mice as described (2.6.2.4). In mesenteric adipose, samples were incubated with protein (300μg) and substrate concentration (10μM) for 24 hours. In subcutaneous adipose, samples were incubated with protein (100μg) and substrate concentration (2μM) for 22 hours.

4.2.7 Biochemical assays

4.2.7.1 Quantification of plasma glucose

Glucose in plasma collected during the glucose tolerance test was quantified by hexokinase assay as described (2.7.2).

4.2.7.2 Quantification of plasma insulin

Insulin in plasma collected during the glucose tolerance test was quantified by ELISA as described (2.7.1).

Tissue	Gene Combination	P value
Liver	TBP	*0.31*
Omental adipose	TBP + Cyc	*0.35*
Mesenteric adipose	Cyc	*0.49*
Subcutaneous adipose	Cyc	*0.81*

Table 4.1 Housekeeping genes used for real-time PCR. TBP = TATA-binding protein; Cyc = cyclophilin. P value indicates results of student's t-test between Vehicle- and Salicylate-treated mice for specific combination of house-keeping genes.

4.2.7.3 Quantification of plasma triglycerides

Fasting plasma triglyceride levels in the plasma collected at the '0' time point during glucose tolerance tests were quantified as described (see 2.7.4).

4.2.7.4 Quantification of plasma NEFAs

NEFAs in plasma collected during the first two time points of the glucose tolerance tests ('0' and '15' min) were quantified as described in (2.7.3).

4.2.8 Statistics

Data are presented as mean ± SEM and were analysed by Student's unpaired t-tests or two-way ANOVA followed by Bonferroni post-hoc tests as appropriate.

4.3 Results

4.3.1 Body and tissue weights

In wild type diet-induced obese (WT-DIO) mice, salicylate reduced weight gain compared to vehicle-treated WT-DIO mice (Table 4.2). In contrast in 11βHSD1-deficient mice fed the same high fat diet (HSD1KO-DIO), salicylate did not alter body weight. In WT-DIO mice, salicylate also significantly altered the weights of several tissues, with reduction in both omental and epididymal adipose depots, as well as a trend towards reduced mesenteric adipose weight. In contrast, salicylate did not alter tissue weights in HSD1KO-DIO mice.

4.3.2 Glucose tolerance test

Plasma glucose and insulin levels were measured during a glucose tolerance test as a measure of insulin sensitivity. Compared to WT-DIO mice, HSD1KO-DIO mice had significantly lower plasma glucose levels (Figure 4.1b). Salicylate treatment in WT-DIO mice improved glucose tolerance as demonstrated by reduced plasma glucose (Figure 4.1b) and insulin (Figure 4.1a) levels. In contrast, salicylate failed to alter plasma glucose or insulin levels in HSD1KO-DIO mice.

4.3.3 Plasma NEFA levels

In WT-DIO mice, salicylate reduced fasting plasma NEFA levels, as well as improving post-prandial NEFA suppression (Figure 4.2). Fasting plasma NEFA levels were not different in HSD1KO-DIO mice compared to WT-DIO, however, post-prandial NEFA suppression was significantly greater in the HSD1KO-DIO group. Salicylate did not alter either fasting or post-prandial plasma NEFA levels in HSD1KO-DIO mice.

Body Weights						
	WT-DIO			HSD1KO-DIO		
	Vehicle	Salicylate	p Value	Vehicle	Salicylate	p Value
Weight at start (g)	38.9 ± 0.7	37.4 ± 0.5	*0.12*	38.0 ± 0.8	38.3 ± 0.5	*0.81*
Weight at end (g)	41.9 ± 0.9	39.2 ± 0.6	***0.02***	38.0 ± 0.8	39.0 ± 0.8	*0.37*
Time on HFD (weeks)	18.5 ± 1.6			10.8 ± 0.8		
Tissue Weights						
	WT-DIO			HSD1KO-DIO		
Tissue	Weight as % Body Weight		p Value	Weight as % Body Weight		p Value
	Vehicle	Salicylate		Vehicle	Salicylate	
Liver	3.90 ± 0.27	3.89 ± 0.22	*0.96*	3.23 ± 0.17	3.39 ± 0.15	*0.47*
S.C. Adipose	1.13 ± 0.12	1.10 ± 0.15	*0.87*	1.29 ± 0.10	1.46 ± 0.12	*0.87*
Omental Adipose	0.12 ± 0.01	0.08 ± 0.01	***0.01***	0.11 ± 0.01	0.12 ± 0.01	*0.52*
Mesenteric Adipose	1.41 ± 0.15	1.03 ± 0.09	***0.05***	1.29 ± 0.08	1.49 ± 0.12	*0.21*
Retroperitoneal Adipose	0.74 ± 0.03	0.59 ± 0.08	*0.15*	0.72 ± 0.03	0.74 ± 0.03	*0.65*
Epididymal Adipose	2.51 ± 0.10	1.97 ± 0.19	***0.02***	2.42 ± 0.09	2.42 ± 0.12	*0.99*
Quadricep Muscle	0.52 ± 0.04	0.57 ± 0.02	*0.25*	0.51 ± 0.05	0.47 ± 0.02	*0.55*
Adrenal	0.009 ± 0.001	0.007 ± 0.001	*0.34*	0.016 ± 0.001	0.008 ± 0.001	*0.32*
Ratio S.C:Omental Adipose	9.6 ± 0.5	15.1 ± 1.3	***0.001***	12.9 ± 1.9	13.9 ± 2.5	*0.75*

Table 4.2 Salicylate-induced changes in body and tissue weights of diet-induced obese wild type mice (WT-DIO) were not observed in diet-induced obese 11βHSD1 knockout mice (HSD1KO-DIO). In WT-DIO mice, salicylate attenuated overall weight gain and reduced weight of omental and mesenteric adipose depots. No significant changes were seen in HSD1KO-DIO mice following treatment. HFD = high-fat diet; S.C. = subcutaneous. Data are mean ± SEM for n=8 per group. Comparisons were by Student's unpaired t-test.

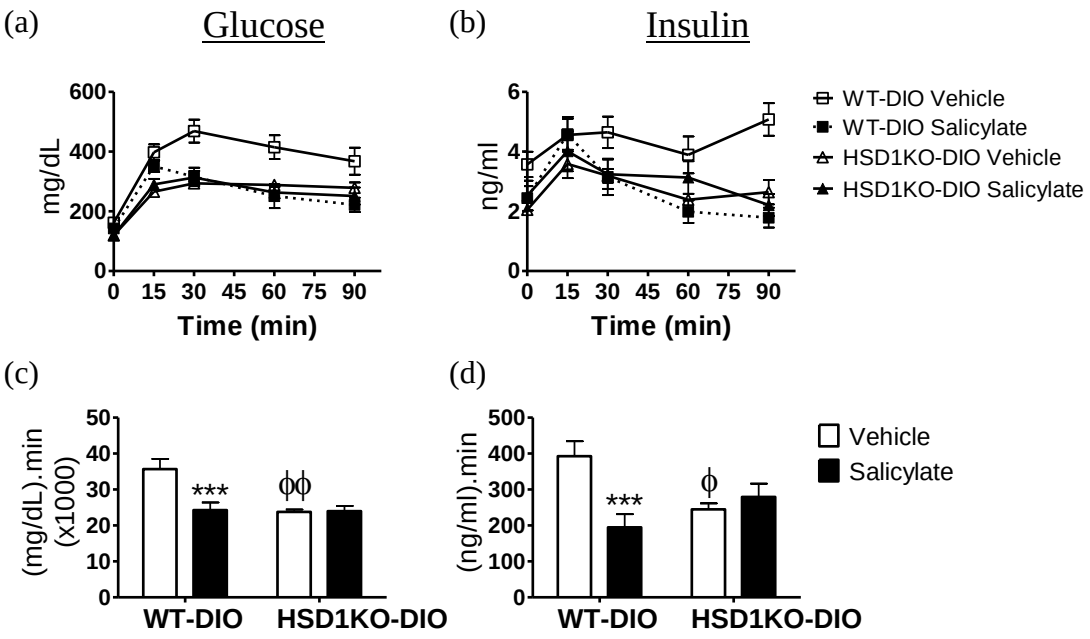

Figure 4.1 Salicylate improved glucose tolerance in WT-DIO mice, but had no effect in HSD1KO-DIO. Plasma glucose (a) and insulin (b) levels over the course of a glucose tolerance test, with areas under the curve (AUC) shown underneath (c and d respectively). Squares denote WT-DIO mice, whilst triangles denote HSD1KO-DIO mice. Open symbols/bars denote vehicle treatment, whilst closed symbols/bars denote salicylate treatment. In WT-DIO mice, salicylate treatment significantly reduced plasma glucose (c) and insulin (d) levels, but had no effect in HSD1KO-DIO mice. HSD1KO-DIO mice had significantly reduced glucose (c) and insulin (d) levels compared to WT-DIO mice. Data are mean ± SEM for n=8 per group for individual time points and for area under the curve. Comparisons for AUC were by two-way ANOVA with Bonferroni post-hoc tests: ***$P<0.05$ vs Vehicle of same genotype; $^{\phi}P<0.05$, $^{\phi\phi}P<0.01$ vs WT-DIO Vehicle.

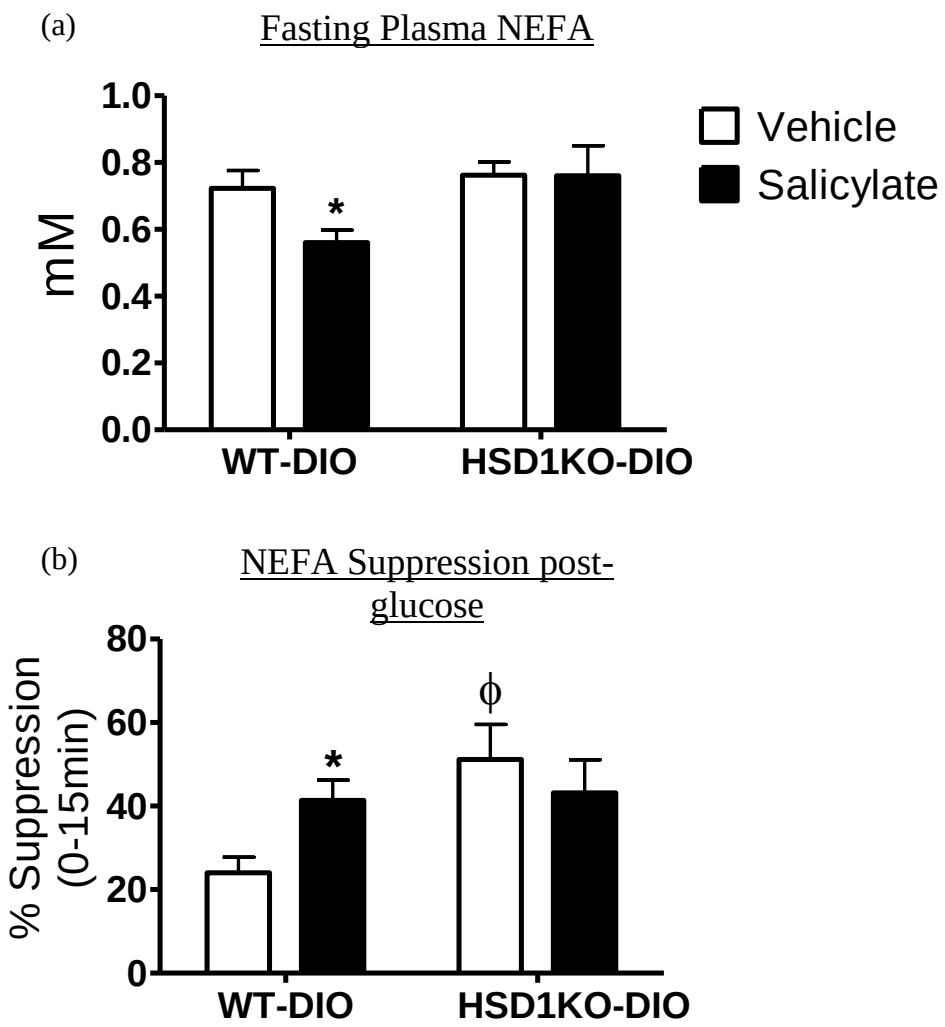

Figure 4.2 Salicylate reduced non-esterified fatty acids (NEFAs) levels in WT-DIO mice, but had no effect in HSD1KO-DIO mice. Fasting plasma NEFA levels (a) and 15 min post-glucose challenge NEFA suppression (b) in diet-induced obese 11βHSD1 knockout mice (HSD1KO-DIO) and diet-induced obese wild type mice (WT-DIO) were measured spectrophotometrically. Open bars denote vehicle treatment, whilst closed bars denote salicylate treatment. Salicylate treatment reduced fasting NEFA levels in WT-DIO mice, but had no effect in HSD1KO-DIO mice (a). Post-glucose challenge NEFA suppression was increased in WT-DIO mice following treatment and whilst HSD1KO-DIO mice had increased NEFA suppression compared to WT-DIO mice, salicylate treatment induced no further changes (b). Data are mean ± SEM for n=8 per group. Comparisons were by two-way ANOVA with Bonferroni post-hoc tests: *P<0.05 vs Vehicle of same genotype; $^{\phi}$ P<0.05 vs WT-DIO Vehicle.

4.3.4 Plasma triglyceride levels

Salicylate had no effect on plasma triglyceride levels in either WT-DIO or HSD1KO-DIO mice, nor were differences observed between genotypes (Figure 4.3).

4.3.5 11βHSD1 expression and activity

In WT-DIO mice, salicylate did not alter mRNA expression of 11βHSD1 in either liver or subcutaneous (S.C.) adipose. A trend towards reduction was seen in omental adipose (*P=0.09)*. However, in mesenteric adipose, salicylate significantly reduced mRNA levels of 11βHSD1 (Figure 4.4). Enzyme activity was measured in mesenteric and S.C. adipose (Figure 4.5). Salicylate significantly reduced enzyme activity in mesenteric adipose, but had no effect in S.C. adipose.

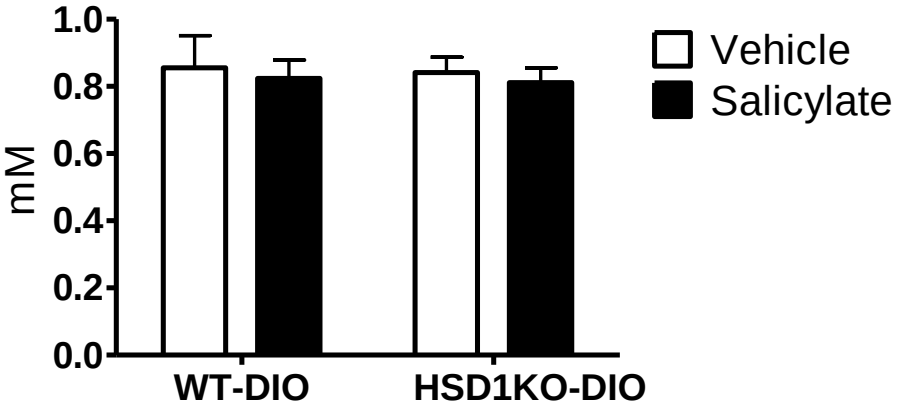

Figure 4.3 Salicylate did not influence triglyceride concentrations in WT-DIO or HSD1KO-DIO mice. Fasting plasma triglyceride levels in diet-induced obese 11βHSD1 knockout mice (HSD1KO-DIO) and diet-induced obese wild type mice (WT-DIO) were measured spectrophotometrically. Data are mean ± SEM for n=8 per group. Comparisons were by two-way ANOVA.

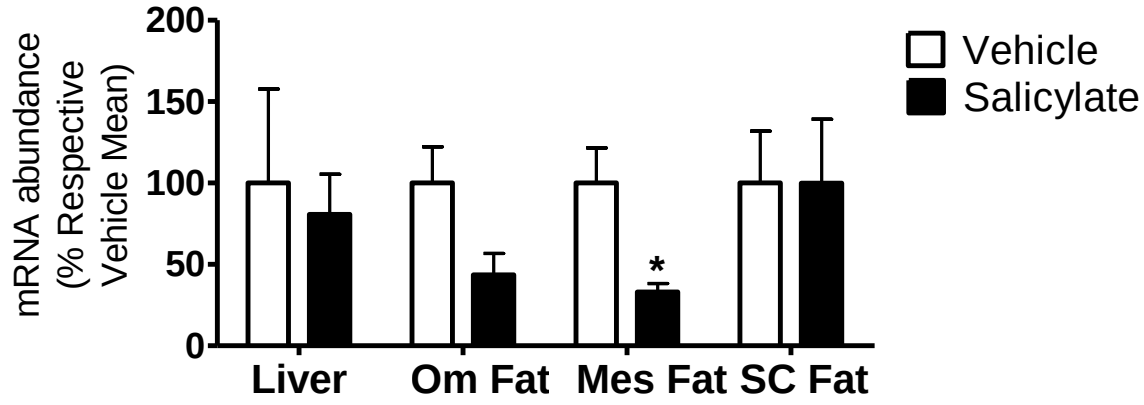

Figure 4.4 Salicylate reduced 11βHSD1 transcript levels in WT-DIO mice. mRNA levels of 11βHSD1 in liver, omental adipose (Om Fat), mesenteric adipose (Mes Fat) and subcutaneous adipose (SC Fat) were determined by real-time PCR. Salicylate (closed bars) significantly downregulated 11βHSD1 in Mes fat compared to vehicle treatment (open bars), with a trend in Om fat (*P=0.09*). Data are mean ± SEM, expressed as % of the respective Vehicle mean. N=6-8 per group (except Om fat salicylate group, n=4). Comparisons were by Student's unpaired t-test: *P<0.05 vs Vehicle.

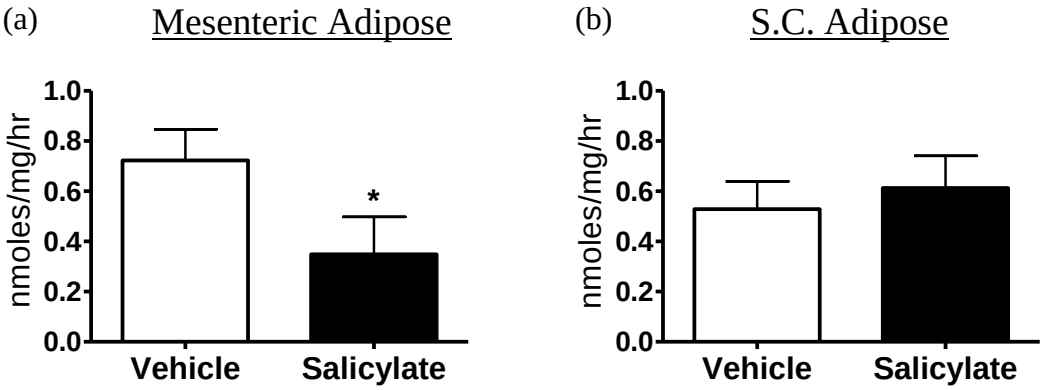

Figure 4.5 Salicylate reduced 11βHSD1 activity in mesenteric adipose of WT-DIO mice. Enzyme activity was measured in mesenteric adipose (a) and subcutaneous (S.C.) adipose (b) and steroids quantified by high performance liquid chromatography. In mesenteric adipose, salicylate (closed bars) significantly reduced 11βHSD1 activity, compared to vehicle treatment (open bars). In S.C. adipose (b), salicylate did not alter enzyme activity. Data are mean ± SEM, expressed as % of the respective Vehicle mean. Nn=7-8 per group. Comparisons were by Student's unpaired t-test: *P<0.05 vs Vehicle.

4.3.6 Adipokine transcript profile

In omental adipose (Figure 4.6), salicylate significantly increased adiponectin and reduced LPL mRNA levels in WT-DIO mice. HSD1KO-DIO mice had significantly higher adiponectin and reduced AGT, TNFα, LPL and ATGL mRNA levels compared to WT-DIO mice. However, salicylate did not induce changes in the abundance of transcripts of these genes in HSD1KO-DIO mice. In mesenteric adipose, similar patterns were seen (Figure 4.7), with salicylate significantly reducing AGT, TNFα, LPL, HSL and ATGL mRNA levels in WT-DIO mice. Only TNFα was significantly different between HSD1KO-DIO and WT-DIO mice, however this may be due reduced n numbers in HSD1KO-DIO groups. As in omental adipose, salicylate did not alter transcript levels in mesenteric adipose of HSD1KO-DIO mice. In S.C. adipose (Figure 4.8), salicylate significantly increased adiponectin in WT-DIO mice. HSD1KO-DIO mice had significantly increased adiponectin compared to WT-DIO mice. However, salicylate had no significant effects on transcript levels in the S.C. adipose of HSD1KO-DIO mice.

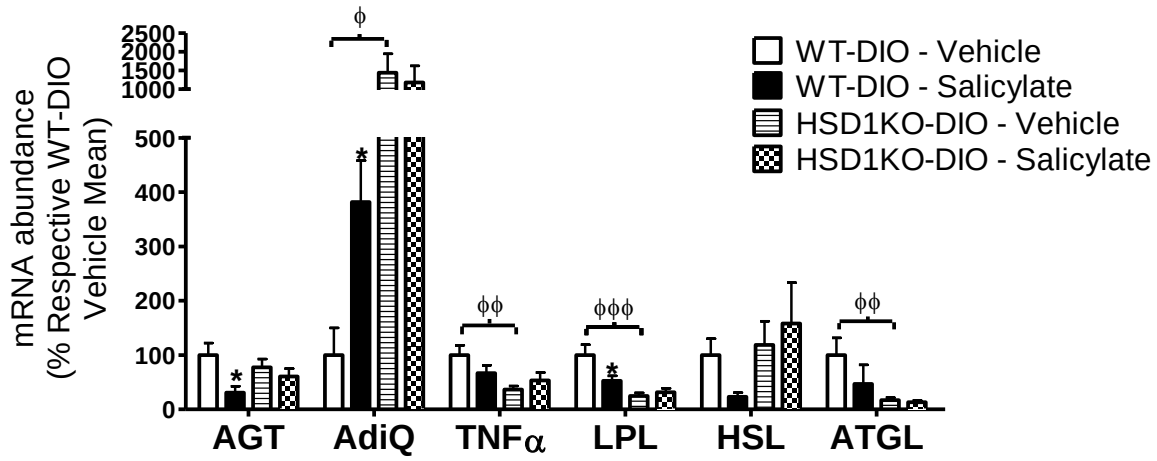

Figure 4.6 Salicylate effects on omental adipose transcript levels in WT-DIO mice are not replicated in HSD1KO-DIO mice. mRNA levels of angiotensinogen (AGT), adiponectin (AdiQ), tumor necrosis factor-α (TNFα), lipoprotein lipase (LPL), hormone sensitive lipase (HSL) and adipose triglyceride lipase (ATGL) were determined by real-time PCR. Salicylate treatment in WT-DIO mice (black bars) resulted in significant downregulation of AGT and LPL, with significant upregulation of AdiQ. HSD1KO-DIO mice (horizontal stripes) had significantly downregulated TNFα, LPL and ATGL, as well as upregulated AdiQ compared to WT-DIO mice (open bars). Salicylate treatment in HSD1KO-DIO mice (hatched bars) had no significant effects. Data are mean ± SEM, expressed as % of the respective WT-DIO Vehicle mean for each gene. N=4-8 per group. Comparisons were by two-way ANOVA with Bonferroni post-hoc tests: *$P<0.05$ vs Vehicle of same genotype; $^{\phi}P<0.05$, $^{\phi\phi}P<0.01$, $^{\phi\phi\phi}P<0.001$ vs WT-DIO Vehicle.

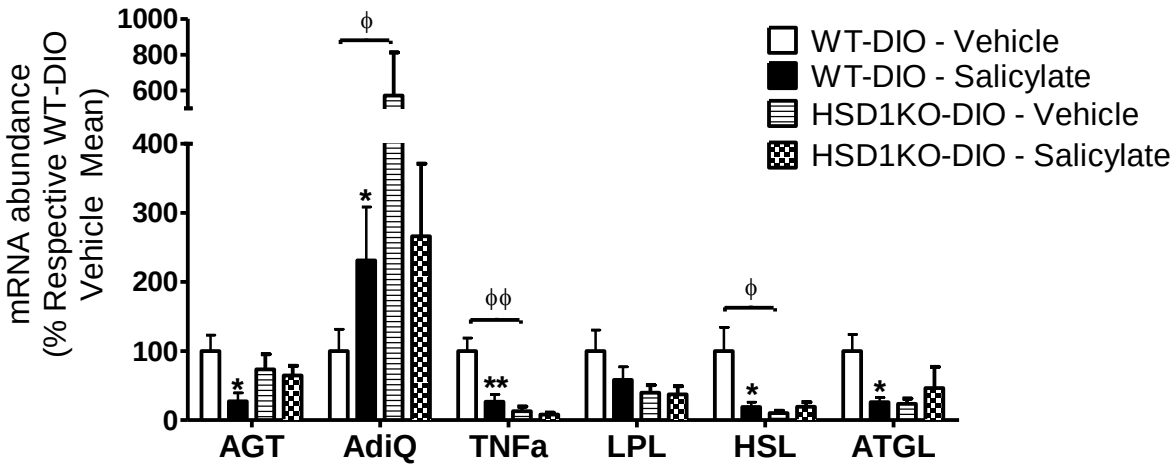

Figure 4.7 Salicylate effects on mesenteric adipose transcript levels in WT-DIO mice are not replicated in HSD1KO-DIO mice. mRNA levels of angiotensinogen (AGT), adiponectin (AdiQ), tumor necrosis factor-α (TNFα), lipoprotein lipase (LPL), hormone sensitive lipase (HSL) and adipose triglyceride lipase (ATGL) were determined by real-time PCR. Salicylate treatment in WT-DIO mice (black bars) significantly downregulated AGT, TNFα, HSL and ATGL. HSD1KO-DIO mice (horizontal stripes) had significantly downregulated TNFα and HSL, as well as upregulated AdiQ compared to WT-DIO mice (open bars). Salicylate treatment in HSD1KO-DIO mice (hatched bars) had no significant effects. Data are mean ± SEM, expressed as % of the respective WT-DIO Vehicle mean for each gene. N=4-8 per group. Comparisons were by two-way ANOVA with Bonferroni post-hoc tests: *P<0.05, **P<0.01 vs Vehicle of same genotype; $^{\phi}$P<0.05, $^{\phi\phi}$P<0.01 vs WT-DIO Vehicle.

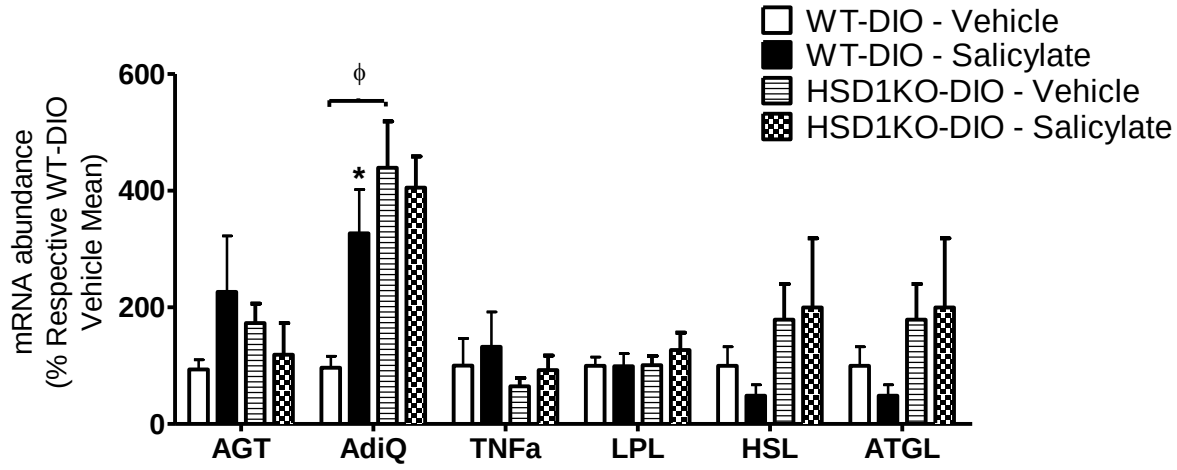

Figure 4.8 Salicylate effects on subcutaneous adipose transcript levels in WT-DIO mice are not replicated in HSD1KO-DIO mice. mRNA levels of angiotensinogen (AGT), adiponectin (AdiQ), tumor necrosis factor-α (TNFα), lipoprotein lipase (LPL), hormone sensitive lipase (HSL) and adipose triglyceride lipase (ATGL) were determined by real-time PCR. Salicylate treatment in WT-DIO mice (black bars) significantly upregulated AdiQ compared to Vehicle (open bars). HSD1KO-DIO mice (horizontal stripes) had significantly upregulated AdiQ compared to WT-DIO mice, but this was not affected by salicylate (hatched bars). Data are mean ± SEM, expressed as % of the respective WT-DIO Vehicle mean for each gene. N=6-8 per group. Comparisons were by two-way ANOVA with Bonferroni post-hoc tests: *P<0.05 vs Vehicle of same genotype; ϕ P<0.05 vs WT-DIO Vehicle.

4.4 Discussion

In this chapter, 11βHSD1-deficient mice were utilised to investigate whether changes in the expression and activity of this enzyme are crucial to the insulin sensitising effects of salicylate in diet-induced obesity. In contrast to wild type mice, these transgenic mice showed no improvements in insulin sensitivity following salicylate treatment. This lack of efficacy supports a key role for downregulation of 11βHSD1 in salicylate's mechanism of action.

Previous reports on 11βHSD1-deficient mice fed a high fat diet demonstrated marked protection to the development of insulin resistance (Kotelevtsev *et al.* 1997; Morton *et al.* 2001; Morton *et al.* 2004). The phenotype displayed in these mice bears a striking resemblance to that seen in the salicylate-treated DIO mice in Chapter 3, with reduced plasma glucose and NEFA levels. As such, it was not unexpected to find a decrease in adipose levels of 11βHSD1 in salicylate-treated mice. Given the evidence that pro-inflammatory mediators are capable of inducing 11βHSD1 expression *in vitro* (Tomlinson *et al.* 2001), one might therefore postulate that salicylate treatment attenuated the inflammation within adipose tissue, which resulted in reduced expression and activity of 11βHSD1. Such a reduction in 11βHSD1 levels would consequently improve the metabolic profile, as has been demonstrated through the use of 11βHSD1 inhibitors (Alberts *et al.* 2002; Alberts *et al.* 2003). However, the data from Chapter 3 only show an association between the effects of salicylate and reduced 11βHSD1 expression. Removing 11βHSD1 from the equation would allow us to determine whether the insulin sensitising effects of salicylate were due specifically to downregulation of the enzyme, or due to an enzyme-independent effect such as reduced pro-inflammatory markers within adipose tissue.

In order to investigate this, 11βHSD1-deficient mice (HSD1KO-DIO) were fed a high-fat diet and weight matched to wild-type C57Bl/6 controls also on a high-fat

diet (WT-DIO). In glucose tolerance tests, it was evident that HSD1KO-DIO mice were more insulin sensitive at baseline than their weight-matched wild-type controls, demonstrated by lower plasma insulin and glucose levels. This was expected, and confirmed the protected metabolic state of 11βHSD1-deficient mice. However, at the outset of the experiment, weight matching was chosen over matching for insulin resistance as the latter would have been difficult to achieve, and weight-matching ensured an obese phenotype. In direct contrast to WT-DIO mice, HSD1KO-DIO mice treated with salicylate exhibited no improvement in glucose tolerance. One could argue that the improved metabolic profile of the HSD1KO-DIO mice may mask any potential benefits of salicylate treatment, as was seen for the 'lean' cohort of mice in Chapter 3. However, if one compares the baseline reading for these mice with the 'lean' cohort of mice from Chapter 3, it is evident that the HSD1KO-DIO mice are not only obese (38.0 ± 0.8 vs 28.8 ± 0.6 g), but hyperinsulinemic in the fasting state (2.01 ± 0.15 vs 0.95 ± 0.11 ng/ml) compared to lean mice. Therefore, it is unlikely that salicylate failed to induce any effects in these mice due to a lack of insulin resistance. In terms of other biochemical improvements in the metabolic profile and supporting the results from Chapter 3, a reduction in fasting plasma NEFA levels was seen in WT-DIO mice following salicylate treatment, whilst no change was observed in HSD1KO-DIO mice. 11βHSD1-deficient mice have previously been reported to have reduced fasting plasma NEFA levels compared to wild type controls (Morton *et al.* 2004). However, this was not observed in this current cohort of mice. Despite this, an increased suppression of plasma NEFA levels was observed post-glucose challenge in HSD1KO-DIO mice compared to WT-DIO mice, indicating improved adipose insulin sensitivity. Similar to fasting levels, NEFA suppression levels were unaltered by salicylate treatment in HSD1KO-DIO mice. The accumulation of this evidence implies that not only is downregulation of 11βHSD1 associated with the salicylate-mediated ability to improve the metabolic profile of DIO mice, but that it is a crucial mediator of this insulin sensitising effect.

Having established the importance of 11βHSD1 in salicylate-induced insulin sensitisation, our focus switched to pro-inflammatory cytokine regulation of this

enzyme. If one is to consider the general hypothesis of what is believed to occur in obese adipose tissue, with marked macrophage infiltration in response to adipocyte hypertrophy (Suganami and Ogawa 2010). The resultant release of pro-inflammatory mediators could act upon adipocytes to drive an increase in 11βHSD1 expression, raising local levels of glucocorticoids and inducing a Cushing's syndrome-like effect. Whilst this is clearly an attractive hypothesis, the main reservation here is the apparent lack of anti-inflammatory effects of glucocorticoids upon the inflammatory state within adipose tissue. Indeed, elevated 11βHSD1 would be expected to dampen any inflammatory response as a result of increasing the local levels of glucocorticoids. In our study, HSD1KO-DIO mice had reduced TNFα expression in visceral adipose compared to WT-DIO mice, indicating a paradoxical situation whereby a reduction in 11βHSD1 actually suppresses inflammation. Furthermore, salicylate treatment in WT-DIO mice reduced visceral expression of TNFα, yet a similar effect was not observed in HSD1KO-DIO mice. These findings raise the possibility that 11βHSD1 is a regulator of the inflammatory response within obese adipose, with increased levels of the enzyme driving inflammation.

To understand this paradoxical situation, it is important to note the effects of exogenous versus endogenous glucocorticoids, as well the distinction in the inflammatory state between the acute response to injury and infection, and the chronic, low grade inflammation seen in obesity. Glucocorticoids at pharmacological doses are used in the treatment of inflammatory conditions (Saklatvala 2002). However, less is known about the physiological effects of endogenous glucocorticoids on inflammation. In response to acute injury and inflammation, glucocorticoids are known to alter leukocyte trafficking and the differentiation of haematopoietic cells (McEwen *et al.* 1997; Ehrchen *et al.* 2007; Tuckermann *et al.* 2007). They also promote the resolution of inflammation by inducing an anti-inflammatory phenotype in differentiating monocytes (Liu *et al.* 1999; Olefsky and Glass 2010). These effects on monocyte/macrophage cells are particularly pertinent when one considers the role these cells play in obesity-linked insulin resistance, given that they are a major source of pro-inflammatory mediators (Fain 2010;

Olefsky and Glass 2010). Indeed, the recent discovery that up to 40% of adipose tissue cell content in obese rodents and humans is composed of macrophages, compared to around 10% in lean adipose, demonstrates the potential impact of these cells (Weisberg *et al.* 2006). Interestingly, the infiltration of macrophages into adipose and the resultant increase in the inflammation precede the development of insulin resistance, indicating the important role that adipose tissue macrophages may play in the pathogenesis of this condition (Weisberg *et al.* 2003; Xu *et al.* 2003). A major advance here was the recent discovery of 11βHSD1 expression in immune cells, in particular that its expression is induced upon differentiation of monocytes to macrophages (Thieringer *et al.* 2001). However, 11βHSD1 expression in macrophages is also dependent upon the polarisation of the specific cell. The classically activated macrophage phenotype (M1) is highly pro-inflammatory and has been shown to have greatly elevated 11βHSD1 mRNA expression and activity compared to the anti-inflammatory alternatively activated phenotype (M2) (Martinez *et al.* 2006). Given that resident adipose tissue macrophages are M2, with the infiltrated macrophages of the M1 phenotype, this demonstrates the pro-inflammatory state of obese adipose tissue (Olefsky and Glass 2010). Furthermore, this highlights that not only adipocytes, but infiltrated macrophages can contribute to the local increase in glucocorticoids within obese adipose tissue through elevated 11βHSD1. However, this still leaves the paradox whereby increased glucocorticoid levels promote rather than suppress the inflammatory response. Recent *in vitro* studies in both macrophages and adipocytes have sought to investigate this, demonstrating that inhibition of 11βHSD1 in both pro-inflammatory stimulated macrophages and pre-adipocytes attenuated the increase in the release of TNFα, IL-6 and IL-1β (Ishii *et al.* 2007; Ishii-Yonemoto *et al.* 2010). This finding indicates that intracellular regeneration of active glucocorticoids by 11βHSD1 exerts pro-inflammatory effects. Indeed, low, physiological, concentrations of glucocorticoids have been shown to induce macrophage expression of pro-inflammatory cytokines, including osteopontin (OPN) (Wang *et al.* 2000) and macrophage migratory inhibitory factor (MIF) (Calandra *et al.* 1995; Leng *et al.* 2009). Furthermore, in TNFα-stimulated pre-adipocytes, overexpression of 11βHSD1 augmented the release of IL-6 and MCP-1 (Ishii-Yonemoto *et al.* 2010). Perhaps most interestingly, this did

not occur in resting pre-adipocytes, indicating that 11βHSD1 only reinforces inflammation under pre-existing inflammatory conditions. This accumulation of data suggests that low, physiological levels of glucocorticoids, such as may be seen as a result of increased 11βHSD1 activity, actually drive inflammation as oppose to suppressing it.

In this chapter, we have shown that salicylates have no beneficial effects in 11βHSD1-deficient mice. This transgenic model is a global knockout and so it is difficult to interpret whether it is the lack of 11βHSD1 in adipose, liver or other tissues that is responsible for 'blocking' the effects of salicylates. However, having seen specific downregulation of visceral adipose levels and activity of 11βHSD1 in wild-type mice following salicylate treatment, it suggests that adipose regulation of 11βHSD1 is crucial to the insulin sensitising effects of anti-inflammatory salicylates. Despite this, as we have seen, adipose tissue does not solely consist of adipocytes, with pre-adipocytes and macrophages found to be present in increasing numbers in obese adipose. As such, it would be of great interest to look more closely at the composition of visceral adipose depots treated with salicylates to determine any changes in immune cell composition and polarisation. One of the many interesting points to arise from this work is the inability of salicylates to suppress inflammatory marker transcript levels in the absence of 11βHSD1, indicating that the inflammatory state in obese adipose tissue is regulated by this enzyme. Whether or not this is determined by adipocyte or macrophage 11βHSD1 remains to be seen, however, the creation of myeloid-specific 11βHSD1 knockout mice would be a great tool in solving this puzzle. What is evident is that 11βHSD1 appears to act as a central pivot in pathways regulating both metabolic and inflammatory signalling, implicating it in playing a causative role in the pathogenesis of obesity-linked insulin resistance. As such, it remains clear that 11βHSD1 is a promising drug target in the global epidemic of obesity.

Chapter 5

The anti-inflammatory mechanisms of 5α-reduced glucocorticoids *in vitro*

5.1 Introduction

Within obesity-associated insulin resistance, it is clear that glucocorticoids play a key role in the development of the metabolic syndrome, with intracellular levels of glucocorticoids altered through metabolising enzymes such as the 11βHSDs. However, another important pathway of glucocorticoid metabolism involves A-ring reduction, and this is mediated primarily by the rate-limiting enzymes in the reaction, 5αR1, and also by 3αHSD, resulting in the generation of 5α-reduced metabolites of glucocorticoids. Interest in the role these enzymes play was sparked when it was discovered that in obesity, there is an increase in hepatic levels of 5αR1 (Livingstone *et al.* 2005). Physiological levels of glucocorticoids have numerous metabolic effects within target cells, as described in previous chapters, with an increase in their levels leading to the development of insulin resistance. The increased reduction of glucocorticoids in the obese setting may represent an adaptive mechanism which limits the exposure of the liver to the metabolic consequences of glucocorticoids. However, given that the full range of effects of these 5α-reduced glucocorticoid metabolites is yet to be established, it is difficult to understand the physiological role of this pathway of glucocorticoid metabolism

The parent glucocorticoids exert their numerous metabolic and immunomodulatory effects primarily via the GR, an intracellular, ligand-activated transcription factor capable of regulating gene transcription through transactivation or transrepression (Saklatvala 2002; De Bosscher *et al.* 2003; Beck *et al.* 2009). Upon ligand-binding, the activated GR dissociates from the heat shock proteins that retain the GR in the cytoplasm in an inactive form. This results in a conformational change that unmasks the nuclear localisation signal, enabling translocation of the GR to the nucleus whereby it acts to induce or repress gene transcription. Previous work in rat hepatocytes identified that the 5α-reduced tetrahydro metabolite of corticosterone, 5αTHB, is capable of displacing dexamethasone from ligand binding sites on GR (McInnes *et al.* 2004). Further work demonstrated that 5αTHB binds and activates GR, inducing translocation to the nucleus (Yang 2009). Following translocation, activated GRs regulate gene transcription through transactivation or transrepression.

The parent glucocorticoid, corticosterone induces transcription of tyrosine aminotransferase (TAT) and phosphoenolpyruvate carboxykinase (PEPCK) in H4IIE hepatoma cells, however there is conflicting evidence on whether 5αTHB induces mRNA expression. One report suggests that this metabolite does induce mRNA expression of TAT and PEPCK (McInnes *et al.* 2004), yet this effect has not been reproducible (Yang 2009).

The potent anti-inflammatory effects of glucocorticoids are widely documented, and the conventional viewpoint is that these effects are believed to involve GR-mediated transrepression (Barnes 1998; De Bosscher and Haegeman 2009; Barnes 2010). In particular, GR-protein interactions with pro-inflammatory transcription factors including NF-κB and AP-1 are believed to suppress production of pro-inflammatory cytokines such as TNFα, IL-6 and MCP-1. However, there is also evidence that glucocorticoids induce expression of anti-inflammatory molecules through transactivation, for example of IL-10 (Clark 2007; Newton and Holden 2007). Previous work has shown that 5αTHB is capable of suppressing release of TNFα and IL-6 from LPS-stimulated bone marrow-derived macrophages, as well as inducing release of IL-10 (Yang 2009). This has led to speculation that 5α-reduced metabolites of corticosterone may act as 'dissociative' steroids, maintaining their anti-inflammatory properties, but having less detrimental side effects such as activation of metabolic pathways. However, the mechanisms behind these anti-inflammatory effects remain unclear. Parent glucocorticoids have been shown to act through blockade of NFκB and AP-1 activation pathways, yet it is unknown whether 5α-reduced metabolites of glucocorticoids, namely 5αDHB and 5αTHB, act in a similar manner, or through a separate mechanism.

Hypothesis

The hypothesis in this chapter is that 5α-reduced metabolites of corticosterone exert their anti-inflammatory effects through inhibition of pro-inflammatory NF-κB and/or AP-1 activation.

Aims

The aims of this chapter were to investigate:

1) Whether 5α-reduced metabolites suppress pro-inflammatory cytokine production and pro-inflammatory signalling pathways.

2) Whether 5α-reduced metabolites inhibit NFκB and AP-1 activation in a GR-dependent manner.

5.2 Materials and methods

5.2.1 Maintenance of cell lines and reagent preparation

5.2.1.1 RAW264.7 (murine macrophage cells)

RAW264.7 cells were cultured as described (2.3.2).

5.2.1.2 HEK293 (human embryonic kidney cells)

HEK293 cells were cultured as described (2.3.1)

5.2.1.3 Preparation of stripped-serum medium

To avoid interference from background concentrations of glucocorticoids found in FBS, stripped serum was prepared. Heat-inactivated FBS (500ml) was mixed with dextran-coated charcoal (5g) and stirred for 16-24 hours at 4°C. The serum was filtered using 5µm and 0.45µm filters (Sartorius, Germany) successively and sterilized by filtration (0.20µm filter (Millipore, MA, USA)). The filtered serum was dispensed into 50ml aliquots and stored at -20°C. The stripped serum medium was prepared with the same components as the normal culture medium for RAW264.7 cells (2.3.2) with the normal FBS replaced by the stripped serum.

5.2.1.4 Preparation of antibiotic-free, stripped-serum medium

Antibiotic-free medium was prepared for use with HEK293 cells undergoing transfection as antibiotics interfere with this process. Stripped serum was prepared as described above (5.2.1.3). The antibiotic-free, stripped serum medium was prepared as described for HEK293 cells (2.3.1) with the normal FBS replaced by the stripped-serum and without the addition of penicillin.

5.2.1.5 Preparation of phenol-red free medium

To avoid spectrophotometric interference in ELISAs, phenol-red free medium was prepared for use with RAW264.7 cells. Phenol-red free DMEM was supplemented with 10% (v/v) heat-inactivated stripped FBS (5.2.1.3), penicillin (100 IU/ml), streptomycin (100μg/ml) and L-glutamine (200mM).

5.2.1.6 Preparation of glucocorticoid treatments

Corticosterone, 5αDHB and 5αTHB were dissolved separately in ethanol to make stock solutions (1M). Solutions were stored at -20°C. A series of dilutions of each glucocorticoid was prepared with ethanol.

5.2.1.7 Preparation of modulators of signalling pathways

Lipopolysaccharide (LPS, 1mg) was suspended in sterile PBS (1ml) to make a stock (1mg/ml) and was stored at -20°C. A series of dilutions was prepared with sterile PBS. 12-O-Tetradecanoyl-phorbol-13-acetate (TPA, 1mg) was suspended in sterile PBS (1ml) to make a stock (1mg/ml) and was stored at -20°C. A series of dilutions was prepared with sterile PBS. The GR antagonist RU486 was dissolved in ethanol to make a stock (1mM).

5.2.2 Suppression of cytokine release in RAW264.7 cells by glucocorticoids

5.2.2.1 Dose response of LPS to induce cytokine release in RAW264.7 cells

The day before the experiment, RAW264.7 cells were plated on 6-well plates at a density of $2\text{-}3\times10^5$ cells per well and incubated in normal medium (2ml) in a humidified atmosphere in 95% air and 5% CO_2 at 37°C.

Twenty four hours later, the medium was removed and the wells washed with sterile PBS (1ml). Phenol-red free stripped-serum medium (5.2.1.5, 2ml) was added to each well and incubated (1 hour) under the above conditions.

Cells were incubated (24 hours) with increasing concentrations of LPS (0, 3, 10, 30, 100ng/ml) before medium was harvested for quantification of cytokine release. Three experiments were performed in duplicate for each dose.

5.2.2.2 Dose response of glucocorticoids to suppress cytokine release in LPS-stimulated RAW264.7 cells

RAW264.7 cells were plated on 6-well plates and incubated (24 hours) as described above (5.2.2.1). Following change of medium to phenol-red free stripped serum medium and incubation (1 hour), cells were treated with increasing concentrations of corticosterone, 5αDHB or 5αTHB (0, 0.01, 0.03, 0.1, 0.3, 1, 3μM for each steroid), with LPS (30ng/ml) or without (PBS, 2μl). Following treatment, cells were incubated (24 hours) under conditions described (2.3.2) before medium was harvested for quantification of cytokine release. Three experiments were performed in duplicate for each dose.

5.2.2.3 GR-mediated suppression of cytokine release

RAW264.7 cells were plated and medium harvested as described above (5.2.2.2), however, one hour prior to glucocorticoid treatment, cells were incubated with the GR antagonist RU486 (1μM). Only one dose of each glucocorticoid (1μM) was used.

5.2.2.4 Quantification of cytokine release

The amount of the inflammatory cytokines TNF-α and IL-6 in the medium of RAW264.7 cells was quantified by ELISA as described (2.6.7).

5.2.3 Suppression of signalling via pro-inflammatory pathways in response to glucocorticoids

5.2.3.1 Treatment of RAW264.7 cells

RAW264.7 cells were plated on 6-well plates and incubated for 24 hours as described above (5.2.2.1). Following change of medium to phenol-red free stripped-serum medium and incubation (1 hour), corticosterone, 5αDHB, 5αTHB (1μM) or Vehicle (100% ethanol, 2μl) were added. Following incubation (1 hour) with steroids, cells were treated with LPS (100ng/ml) and incubated (30 min) before being lysed and protein extracted (2.5.2.2).

5.2.3.2 Assessment of protein levels in RAW264.7 cells

Following quantification of protein levels from total cell lysates (2.5.2.3), amounts of specific proteins were assessed by Western blotting (2.5.2.5). Primary antibodies directed against IκBα, NFκB, JNK, phospho-JNK, p38 and phospho-p38, all raised in rabbit, were from Cell Signalling Technology (New England Biolabs, Hertfordshire, UK). The antibody directed against MKP-1 (raised in rabbit) was from Santa Cruz Biotechnology (Insight Biotechnology, Middlesex, UK). The antibody directed against β-tubulin (raised in mouse) was from Millipore (Hertfordshire, UK). Secondary fluorescent antibodies directed against rabbit and mouse primary antibodies, both raised in goat, were from LI-COR Biosciences. Protein bands of interest were visualised using the Licor Odyssey (2.5.2.5.4).

5.2.4 Suppression of pro-inflammatory transcription factor activity in response to glucocorticoids

5.2.4.1 Transcription factor plasmids

The luciferase reporter plasmids for NFκB (3x(NFκB)tk-luc) and AP-1 (-517/+63col-luc) (2.7.1) were grown as described (2.7.2)

5.2.4.2 Transfection of RAW264.7 cells

Initially, transfection of RAW264.7 cells with luciferase reporter plasmid for NFκB and AP-1 was attempted. However, preliminary experiments using the protocol described (2.7.3), revealed that positive control plasmids were not producing luciferase activity greater than 'empty' negative control plasmids. A number of optimisation methods were undertaken to overcome this:

- The volume of FuGENE HD transfection reagent added to the transfection complex was altered. The FuGENE HD reagent forms a complex with the DNA and then transports this complex into the cells. Therefore, altering the ratio of FuGENE:DNA impacts on complex composition and function. The protocol described in 2.7.3 used a FuGENE:DNA ratio of approximately 2:1. In attempting to transfect RAW264.7 cells, the following ratios were tried: 3:2, 4:2, 5:2, 6:2, 7:2 and 8:2.

- The volume of the transfection complex added to each well was altered. The protocol described in 2.7.3 required addition of 50µl transfection complex to each well. The following volumes of transfection complex were tested: 75µl, 100µl, 150µl and 200µl.

- The incubation time for complex formation was altered. In the protocol described in 2.7.3, the transfection complex was added immediately to the cells. In attempting to optimise transfection of RAW264.7 cells, the following incubation times were tested for the transfection: 15 min, 30 min, 1 hour (all RT).

Despite these attempts, successful transfection was not achieved and so another cell line previously used to transfect these plasmids was used as an alternative (Bladh *et al.* 2005).

5.2.4.3 Transfection of HEK293 cells

Transfection of HEK293 cells was as described (2.4.3).

5.2.4.3 Glucocorticoid treatment of TPA-stimulated HEK293 cells

Twenty four hours after transfection, HEK293 cells were incubated with TPA (5ng/ml) or vehicle (PBS, 2µl) to stimulate pro-inflammatory transcription factor-mediated luciferase activity. Cells were co-incubated with corticosterone, 5αDHB or 5αTHB (1µM) (24 hours) before luciferase activity was quantified. Three experiments were performed in duplicate.

5.2.4.4 Quantification of luciferase activity

Luciferase activity of transfected HEK293 cells was quantified as described (2.7.4).

5.2.4.5 Data analysis

Data for the transient transfection study were analysed using the raw data of luminescent intensity of the luciferase assay. The values of the luciferase activity were calculated by subtraction of the corresponding mean values of the background that were produced by transfections with pGEM only. In order to normalise for transfection efficiency, a *LacZ* plasmid (encoding β-galactosidase) under the control of the simian virus-40 (SV40) promoter was co-transfected into HEK293 cells. However, it was found that TPA-stimulation significantly induced expression of β-galactosidase and so was not a viable transfection control. A switch was made to the Dual Luciferase Assay kit (Promega, Southampton, UK), which utilised a *Renilla* luciferase reporter plasmid under the control of the cytomegalovirus (CMV) promoter as an internal control for transfection efficiency. However, TPA treatment here also resulted in an upregulation of *Renilla* luciferase activity. For this reason, transfection efficiency was not controlled using an internal control plasmid. Instead, all experiments were performed in triplicate, and only deemed acceptable if difference from mean for triplicates was <15%. Transfection was only deemed acceptable if the luciferase activity of the positive control plasmid was >500 times greater than the 'empty' plasmid negative control.

5.2.5 Assessment of A-ring reductase expression in RAW264.7 cells

5.2.5.1 Treatment of cells

RAW264.7 cells were plated on 6-well plates and incubated (24 hours) as described above (5.2.2.1). Cells were washed with sterile PBS and incubated (1 hour) in their respective growth medium containing stripped-serum instead of normal serum. Cells were treated with LPS (30ng/ml) or vehicle (PBS) and incubated (24 hours).

5.2.5.2 Molecular biology

Total RNA was extracted from plated cells (2.6.1.3) before quantification of RNA (2.6.1.4). First strand cDNA was synthesised by reverse transcription (2.6.1.6). Expression of 5αR1, 5α-reductase-2 (5αR2), 5β-reductase (5βR), 3αHSD and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were assessed following PCR (2.6.1.7).

5.2.6 Statistics

Data are presented as mean ± SEM and were analysed by One-way Analysis of Variance (ANOVA) with post-hoc tests as appropriate.

5.3 Results

5.3.1 Cytokine release in LPS-stimulated RAW264.7 macrophages: LPS dose-response

LPS-stimulation of RAW264.7 cells resulted in a dose-dependent increase in the release of both TNFα (Figure 5.1a) and IL-6 (Figure 5.1b). EC50 values of 53.89ng/ml for TNFα and 44.41ng/ml for IL-6 were calculated. A submaximal dose of 30ng/ml was chosen to assess the effects of steroids upon cytokine release in subsequent experiments.

5.3.2 Suppression of cytokine release in LPS-stimulated RAW264.7 cells by glucocorticoids

LPS stimulation of RAW264.7 cells resulted in an increase in the levels of both TNFα (Figure 5.2a-c) and IL-6 (Figure 5.2d-f) above control. Corticosterone (B), 5αDHB and 5αTHB suppressed release of both TNFα and IL-6 in a dose-dependent manner. However, at equimolar concentrations, 5αDHB had a significantly weaker suppressive effect on TNFα release than both B and 5αTHB In terms of IL-6 suppression, at equimolar concentrations, 5αTHB had a weaker suppressive effect compared to both B and 5αDHB.

5.3.3 Effects of GR antagonism on suppression of cytokines by steroids in LPS-stimulated RAW264.7 cells

The GR antagonist RU486 was incubated with a single concentration (1μM) of each steroid and TNFα and IL-6 levels assessed (Figure 5.3a and 5.3b respectively). Co-incubation with the GR antagonist prevented any reduction in cytokine release by B, 5αDHB or 5αTHB.

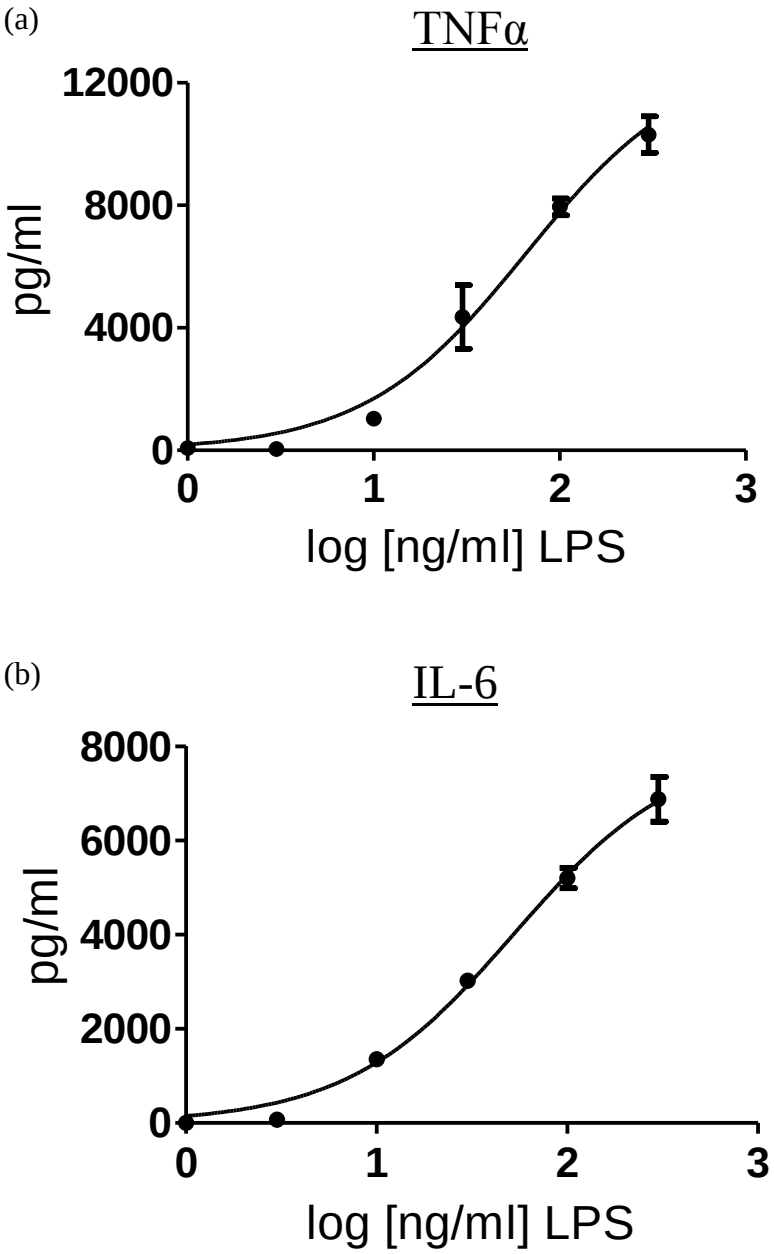

Figure 5.1 Dose-dependent increase in cytokine release in LPS-stimulated RAW264.7 macrophages. RAW264.7 cells were incubated with increasing concentrations of lipopolysaccharide (LPS) for 24 hours and cytokine release into culture media quantified by ELISA. LPS-stimulation resulted in a dose-dependent increase in TNFα (a) and IL-6 (b) release. Data are mean ± SEM for n=3 in duplicate per group.

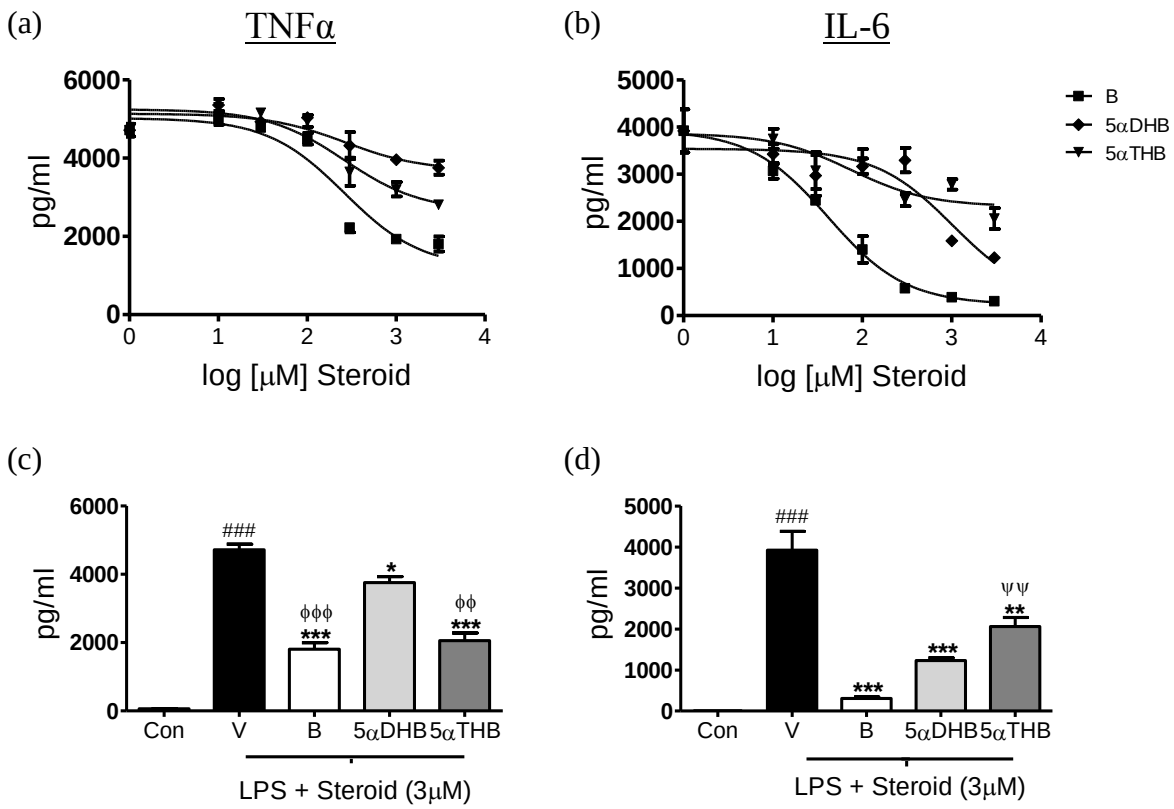

Figure 5.2 Glucocorticoids suppress cytokine release from LPS-stimulated macrophages. RAW264.7 cells stimulated with lipopolysaccharide (LPS, 30ng/ml) or control (PBS, Con) were incubated with increasing concentrations of corticosterone (B), 5α-dihydrocorticosterone (5αDHB) or 5α-tetrahydrocorticosterone (5αTHB) for 24 hours prior to quantification of TNFα (a) and IL-6 (b). Suppressive effects of the highest dose of each steroid are shown for TNFα (c) and IL-6 (d). Data are mean ± SEM for n=3 in duplicate per group. Comparisons (c, d) were by one-way ANOVA with Tukey post-hoc tests. $^{###}P<0.001$ vs Con; *P<0.05, **P<0.01, ***P<0.001 vs V (vehicle); $^{\psi\psi}P<0.01$ vs B; $^{\phi\phi}P<0.01$, $^{\phi\phi\phi}P<0.001$ vs 5αDHB.

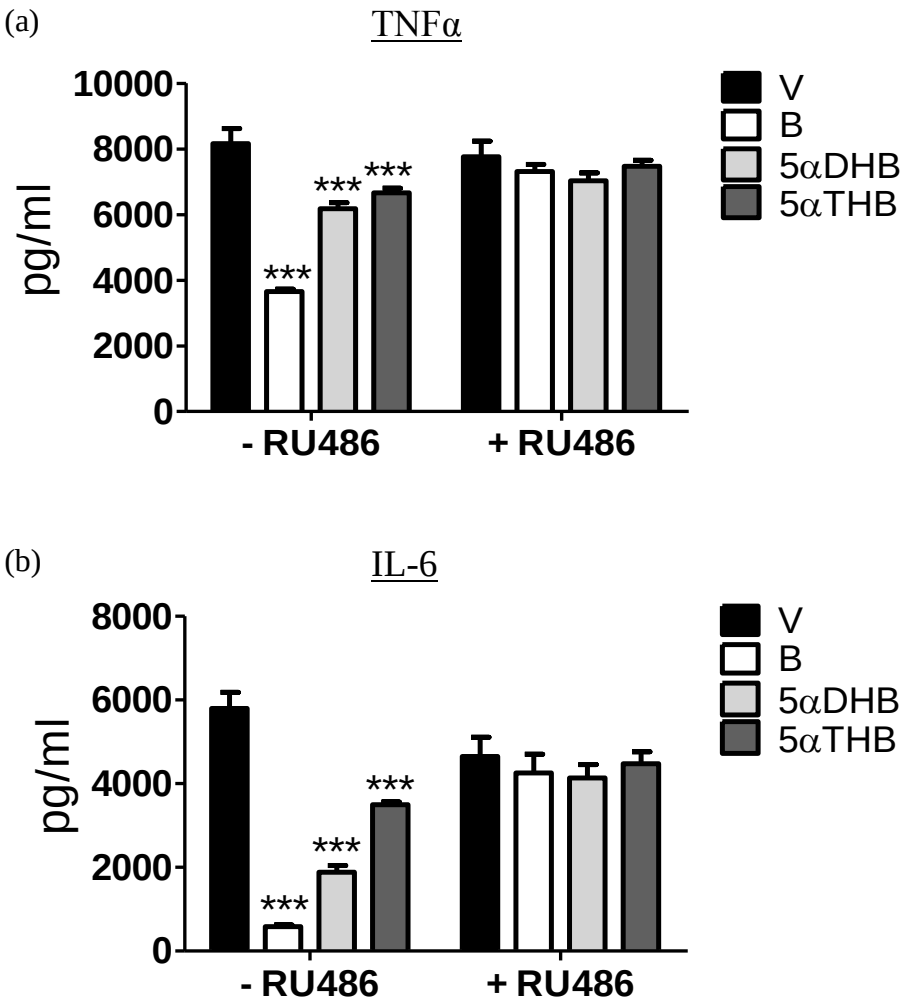

Figure 5.3 Effects of steroids in LPS-stimulated RAW264.7 macrophages are blocked by GR antagonism. RAW264.7 cells were incubated with or without the glucocorticoid receptor (GR) antagonist RU486 (1μM) (+RU486 and –RU486 respectively) for 1 hour prior to treatment with corticosterone (B, 1μM), 5α-dihydrocorticosterone (5αDHB, 1μM), 5α-tetrahydrocorticosterone (5αTHB, 1μM) or Vehicle (ethanol). 1 hour after steroid treatment, cells were stimulated with lipopolysaccharide (LPS, 30ng/ml) for 24 hours prior to quantification of cytokine levels by ELISA. B, 5αDHB and 5αTHB suppressed TNFα (a) and IL-6 (b) in the absence of the GR antagonist. Suppression of TNFα (a) or IL-6 (b) was not observed in the presence of the GR antagonist. Data are mean ± SEM for n=3-6 per group. Comparisons were by Two-way ANOVA with Bonferroni post-hoc tests. ***P<0.001 vs respective vehicle (V).

5.3.4. Protein abundance and phosphorylation states in glucocorticoid-treated LPS-stimulated RAW26.7 macrophages

LPS stimulation resulted in an increase in the phosphorylation of both JNK (Figure 5.4a) and p38 (Figure 5.4b) compared to control. Treatment with B resulted in a significant suppression of both JNK and p38 phosphorylation. Similar to B, both 5αDHB and 5αTHB also suppressed JNK and p38 phosphorylation (Figure 5.4). LPS-stimulation reduced expression of IκBα (Figure 5.5a) and mitogen-activated protein kinase phosphatase 1 (MKP-1) (Figure 5.5b). All steroids increased the amount of MKP-1 compared to vehicle. Whilst both B and 5αDHB increased the amount of IκBα, 5αTHB had no significant effect.

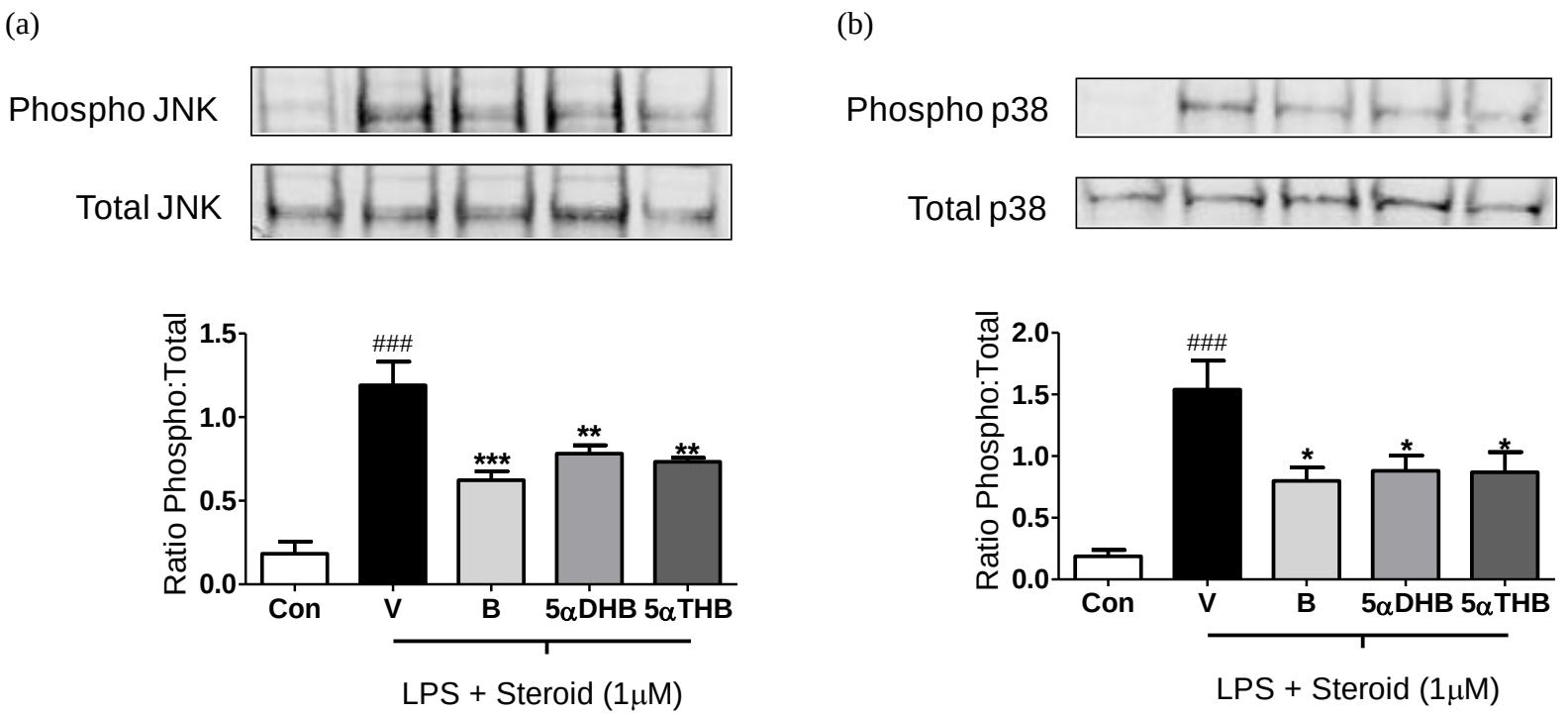

Figure 5.4 5α-Reduced metabolites suppress JNK and p38 phosphorylation. Total and phosphorylated protein levels of the c-Jun N-terminal kinase (JNK) (a) and p38 kinase (b) were analysed in lipopolysaccharide (LPS) stimulated RAW264.7 macrophages. Steroids were administered 1 hour prior to LPS stimulation and cells harvested for protein 30 min later. Representative blot of single experiment shown above graphs. Both 5αDHB and 5αTHB suppressed phosphorylation of JNK and p38 to a similar extent to corticosterone (B), compared to Vehicle (V) treatment. Data are mean ± SEM for n=4 per group. Comparisons were by one-way ANOVA with Dunnett's post-hoc tests. $^{###}P<0.001$ vs Con; *P<0.05, **P<0.01 vs Vehicle (V).

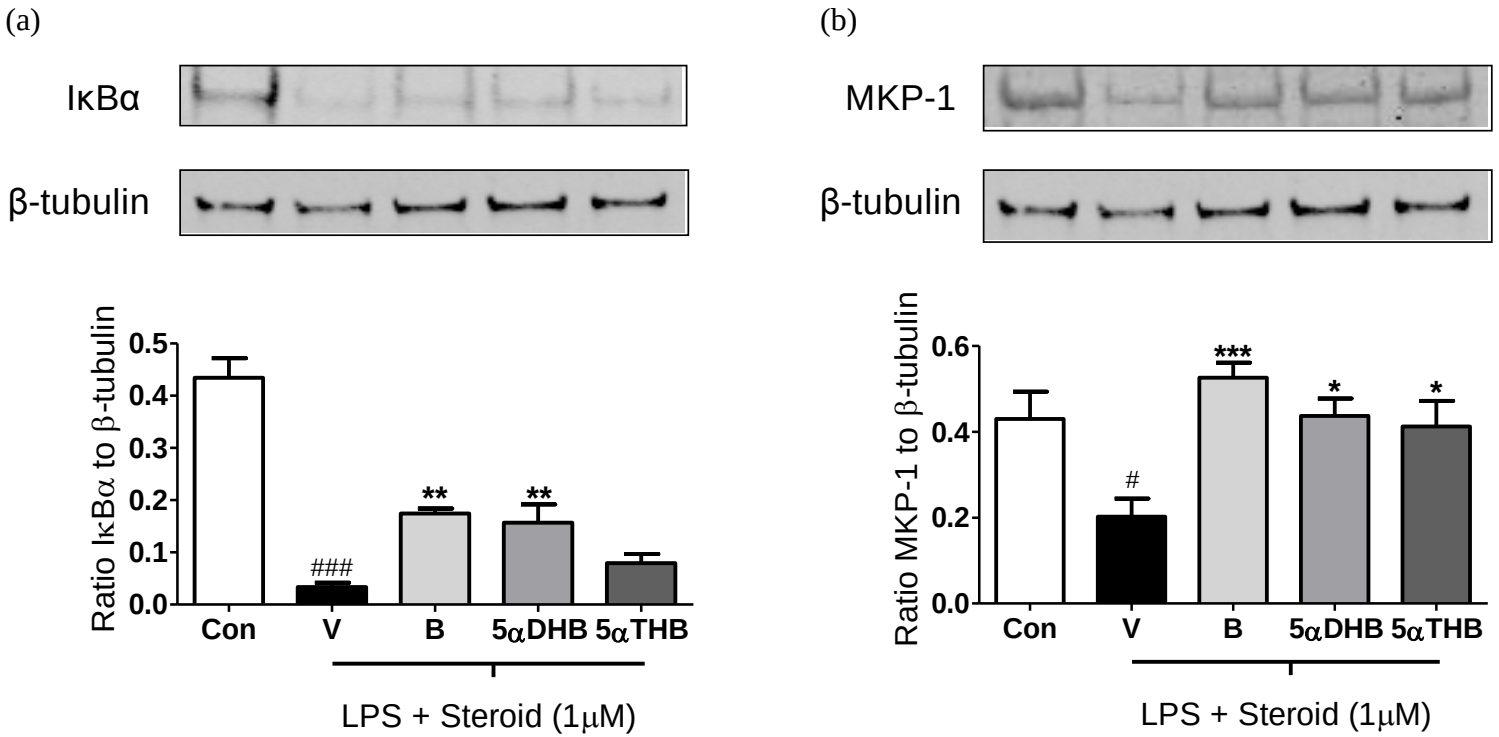

Figure 5.5 Both 5α-Reduced metabolites induce MKP-1 expression, but 5αDHB induces IκBα expression. Protein levels of IκBα (a) and MKP-1 (b) were analysed in lipopolysaccharide (LPS) stimulated RAW264.7 macrophages. Steroids were administered 1 hour prior to LPS stimulation and cells harvested for protein 30 min later. Representative blot of single experiment shown above graphs. Both corticosterone (B) and 5αDHB increased the amount of IκBα, whilst 5αTHB had no effect (a). All steroids increased amount of MKP-1 (b). Data are mean ± SEM for n=5 per group. Comparisons were by one-way ANOVA with Dunnett's post-hoc tests. $^{###}$P<0.001 vs Con; *P<0.05, **P<0.01 vs Vehicle (V).

5.3.5 Suppression of NFκB activation in TPA-stimulated HEK293 cells by glucocorticoids

Phorbol ester (TPA) stimulation of HEK293 cells resulted in a significant increase in NFκB-mediated luciferase activity (Figure 5.6a) compared to control. In HEK293 cells co-transfected with GR (Figure 5.6a), both B and 5αDHB significantly suppressed NFκB-mediated luciferase activity. However, 5αTHB increased NFκB-mediated luciferase activity. In HEK293 cells not co-transfected with GR, the ability of B and 5αDHB to suppress NFκB-mediated luciferase activity was lost, however, 5αTHB retained the ability to increase NFκB-mediated luciferase activity.

5.3.6 Suppression of AP-1 activation in TPA-stimulated HEK293 cells by glucocorticoids

Phorbol ester (TPA) stimulation of HEK293 cells resulted in a significant increase in AP-1-mediated luciferase activity (Figure 5.7a) compared to control. Both B and 5αDHB suppressed AP-1-mediated luciferase activity in HEK293 cells co-transfected with GR, whilst 5αTHB increased AP-1-mediated luciferase activity. The suppressive ability of B and 5αDHB on AP-1-mediated luciferase activity was lost in HEK293 cells not co-transfected with GR. However, 5αTHB retained the ability to increase AP-1-mediated luciferase activity (Figure 5.7b).

5.3.7 Expression of A-ring reductases in RAW264.7 cells

The presence of 5α-Reductase-1 (5αR1), 5α-Reductase-2 (5αR2), 5β-Reductase (5βR) and 3α-hydroxysteroid dehydrogenase (3αHSD) mRNA was assessed in RAW264.7 cells (Figure 5.8). Only 5αR1 was transcribed in RAW264.7 cells (Figure 5.8a), whilst none of the other genes were transcribed. Presence of a house-keeping product, GAPDH (Figure 5.8), ensured intact RNA and successful PCR.

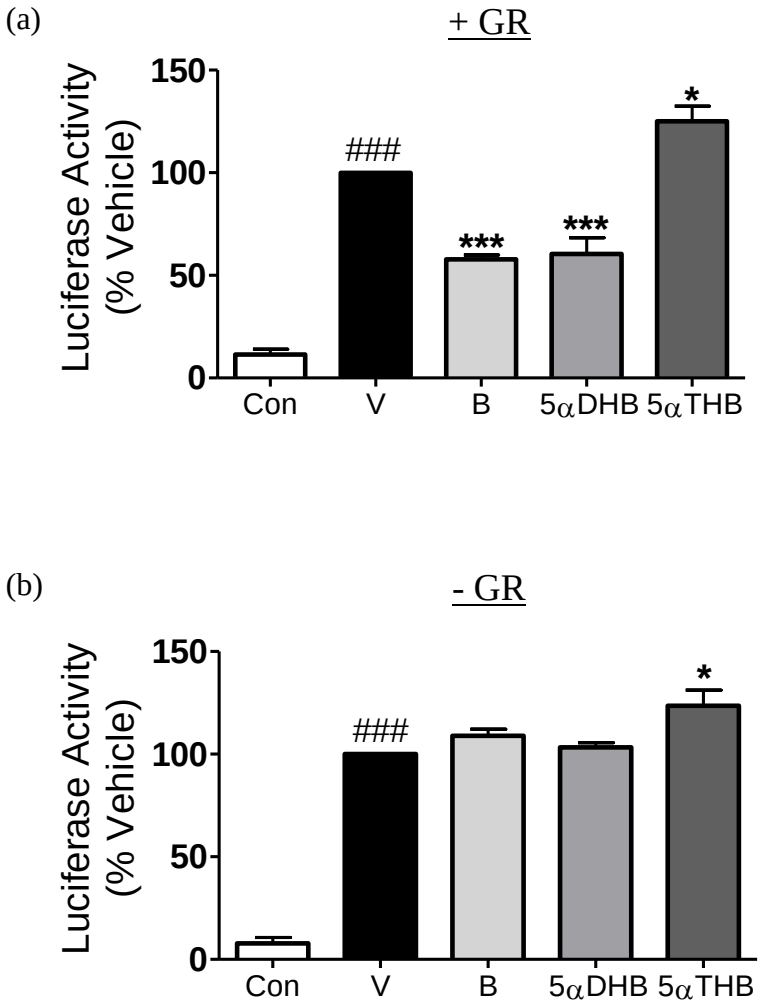

Figure 5.6 Differential effects of steroids on NFκB-mediated luciferase activity in transfected HEK293 cells stimulated with TPA. HEK293 cells were co-incubated with phorbol ester (TPA) or control (phosphate buffered saline, Con) and corticosterone (B, 1μM), 5α-dihydrocorticosterone (5αDHB, 1μM), 5α-tetrahydro-corticosterone (5αTHB, 1μM) or Vehicle (ethanol) for 24 hours. HEK293 cells were studied with glucocorticoid receptor (GR) co-transfected (+GR) (a) and without GR co-transfection (-GR) (b). B and 5αDHB suppressed NFκB-mediated luciferase activity in cells with GR present, however, 5αTHB increased activity (a). In cells without GR, the suppressive effects of B and 5αDHB were not seen. However, 5αTHB maintained the ability to increase NFκB-mediated luciferase activity (b). Data are mean ± SEM for n=3 in triplicate per group. Comparisons were by two-way ANOVA with Bonferroni post-hoc test. $^{###}P<0.001$ vs Con; $*P<0.05$, $***P<0.0001$ vs respective Vehicle.

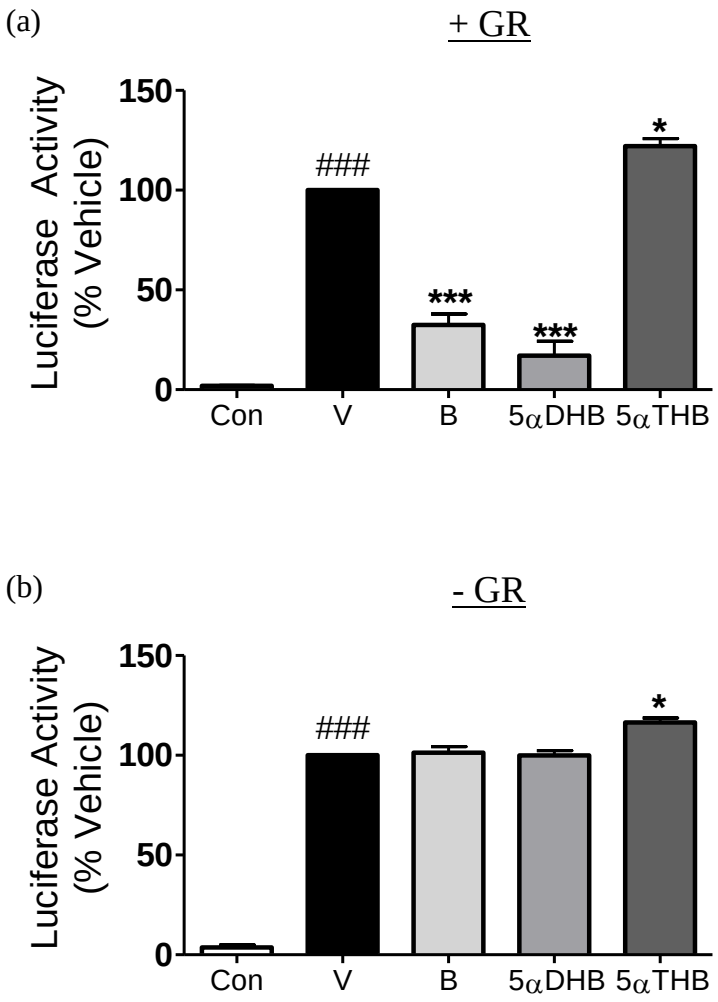

Figure 5.7 Differential effects of steroids on AP-1-mediated luciferase activity in transfected HEK293 cells stimulated with TPA. HEK293 cells were co-incubated with phorbol ester (TPA) or control (phosphate buffered saline, Con) and corticosterone (B, 1μM), 5α-dihydrocorticosterone (5αDHB, 1μM), 5α-tetrahydro-corticosterone (5αTHB, 1μM) or Vehicle (ethanol) for 24 hours. HEK293 cells were studied with (+GR) (a) and without (-GR) (b) glucocorticoid receptor (GR) co-transfected. In HEK293 cells with GR present (a), B and 5αDHB suppressed AP-1-mediated luciferase activity in cells However, 5αTHB increased activity. In HEK293 cells without GR (b), 5αTHB maintained the ability to increase AP-1-mediated luciferase activity, whilst B and 5αDHB lost their suppressive ability. Data are mean ± SEM for n=3 in triplicate per group. Comparisons were by two-way ANOVA with Bonferroni post-hoc tests. $^{###}P<0.001$ vs Con; $*P<0.05$, $***P<0.0001$ vs respective Vehicle.

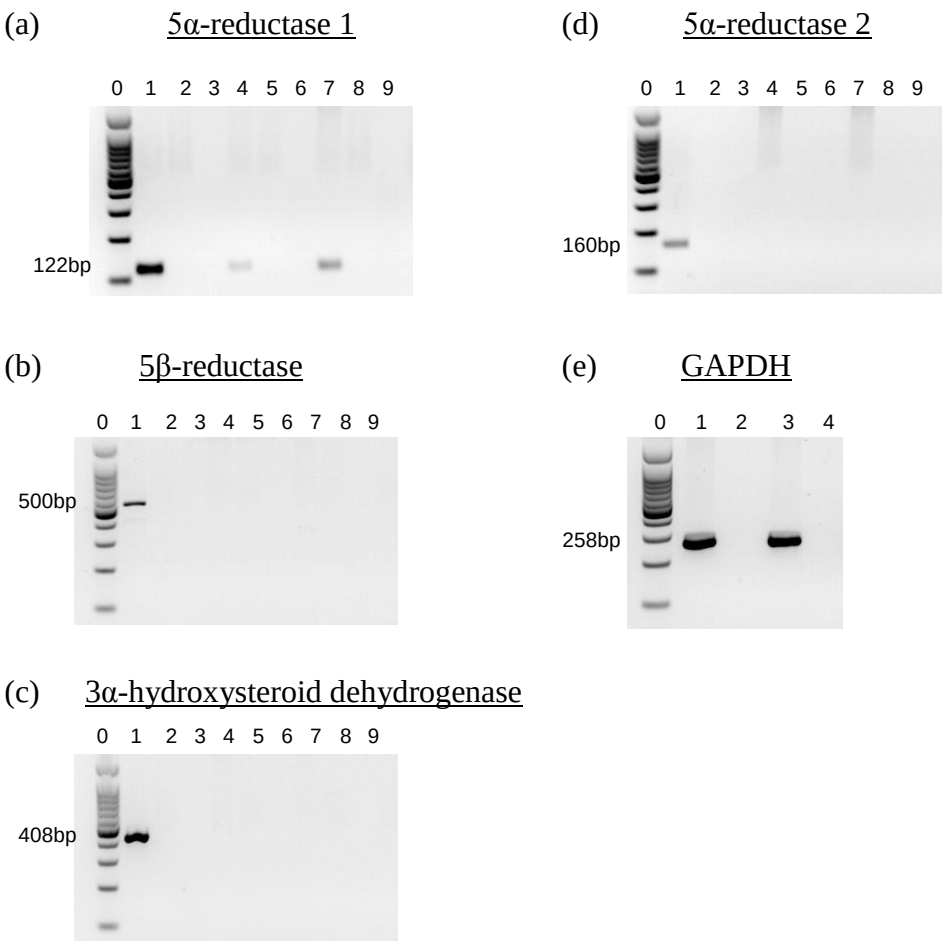

Figure 5.8 Expression of A-ring reductases in RAW264.7 cells. The presence of mRNA for 5α-Reductase-1 (a), 5β-Reductase (b), 3α-hydroxysteroid dehydrogenase (c) and 5α-Reductase-2 (d) were assessed in RAW264.7 cells. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (e) was assessed as a positive control for successful PCR reaction. 5α-Reductase-1 was expressed in both unstimulated and lipopolysaccharide (LPS)-stimulated RAW264.7 cells (a, lanes 4 and 7 respectively).

Lane setup (a-d):

0 = DNA Ladder
1 = positive control (a-c, liver; d, prostate)
2,3= negative controls for 1
4 = unstimulated-RAW264.7 cells
5,6 = negative controls for 4
7 = LPS-stimulated RAW264.7 cells
8,9 = negative controls for 7

Lane setup (e):

0 = DNA Ladder
1 = unstimulated-RAW264.7 cells
2 = negative control (no cDNA)
3 = LPS-stimulated RAW264.7 cells
4 = negative control (no cDNA)

5.4 Discussion

In this chapter, *in vitro* inflammatory systems were used to investigate the cellular mechanisms underpinning the effects of 5α-reduced metabolites of corticosterone, with the effects summarised in Table 5.1. In LPS-stimulated RAW264.7 macrophages, both 5αDHB and 5αTHB suppressed release of the pro-inflammatory cytokines TNFα and IL-6 in a dose-dependent manner. Pre-treatment with the GR antagonist RU486 ameliorated the suppressive effects of all steroids, indicating anti-inflammatory actions through GR. The cellular mechanisms underpinning the anti-inflammatory effects of 5α-reduced metabolites in macrophages were investigated by analysing inflammatory signalling pathways. LPS-mediated phosphorylation of both JNK and p38 was inhibited by 5αDHB and 5αTHB. Transactivation of MKP-1 was observed following 5αDHB and 5αTHB treatment. However, of the two 5α-reduced metabolites, only 5αDHB increased the amount of IκBα. To establish if these anti-inflammatory effects were a result of inhibition of pro-inflammatory transcription factor activation, HEK293 cells were transiently transfected with luciferase reporter plasmids for NF-κB and AP-1. Following stimulation with TPA, 5αDHB suppressed NF-κB and AP-1-activation to a similar level to corticosterone. However, 5αTHB increased both NF-κB and AP-1 activation. The effects of 5αDHB in HEK293 cells was found to be GR-dependent, however, those of 5αTHB were GR-independent.

Metabolism of steroids represents a mechanism by which tissues can regulate exposure to, and subsequent action of, a range of steroids. In particular, A-ring reduction of steroids provides an intriguing pathway by which endogenous steroids are metabolised. The presence of 5α-reductases and 3α-hydroxysteroid dehydrogenase means that steroids possessing a double bond at the $\Delta^{4,5}$ position may first be 5α-reduced and then 3α-hydroxylated. In terms of glucocorticoids, it was, until recently, believed that such metabolism was a pathway of inactivation. However, evidence from other steroids suggested this may not be the case. Indeed, the role of 5α-reduction was first discovered in androgen physiology, in which it was

Cell type	Effect	B	GR-dependent	5αDHB	GR-dependent	5αTHB	GR-dependent
Macrophage (RAW264.7)	Suppression of pro-inflammatory cytokine release	✓	Yes	✓	Yes	✓	Yes
	Suppression of MAPK inflammatory signalling	✓		✓		✓	
	Induction of MKP-1	✓		✓		✓	
	Induction of IκBα	✓		✓		✘	
Kidney (HEK293)	Suppression of NFκB activation	✓	Yes	✓	Yes	✘	No
	Suppression of AP-1 activation	✓	Yes	✓	Yes	✘	No

Table 5.1 Summary of *in vitro* effects of corticosterone and its 5α-reduced metabolites. Highlighted are the effects of each corticosterone (B), 5α-dihydrocorticosterone (5α DHB) and 5α-tetrahydrocorticosterone (5αTHB) on various inflammatory mechanisms in RAW264.7 murine macrophages and the HEK293 human kidney cells. The column directly adjacent to each steroid depicts if the effect was tested to be dependent upon the action of the glucocorticoid receptor (GR). ✓= positive effect, ✘ = negative effect, <blank> = not tested.

found that 5α-dihydro-testosterone (5αDHT), the 5α-reduced form of testosterone (T), binds to the androgen receptor with a greater affinity than T (Siiteri and Wilson 1974). Further work identified that whilst T is the principal androgen, the active androgen in many tissues is 5αDHT (George 1997). 5α-Dihydro-progesterone (5αDHP), the 5α-reduced form of progesterone (P), is also capable of exerting effects via the progesterone receptor (Muller and Kerschbaum 2006). In microglial cells, 5αDHP has been shown to suppress LPS-induced nitric oxide release, as has the tetrahydro form of P, 5αTHP. Such work stimulated interest in the study of 5α-reduced glucocorticoids and their potential to exert similar actions to their parent steroid. Initial work revealed that both 5αDHB and 5αTHB bind to the GR, with 5αTHB having a greater binding affinity (McInnes *et al.* 2004). This led to further investigation into 5αTHB as an activator of GR. In HEK293 cells, it was found that 5αTHB translocated GR into the nucleus, although at a significantly slower rate than B (Yang 2009). Unlike B, 5αTHB failed to transactivate several glucocorticoid responsive genes in these cells, namely PEPCK and TAT, suggesting an inability to fully activate GR (Yang 2009), although this is in contrast to a previous report suggesting an ability to transactivate TAT and PEPCK (McInnes *et al.* 2004). Despite this, further work into this metabolite revealed that 5αTHB was in fact capable of suppressing pro-inflammatory cytokine release in LPS-stimulated bone-marrow derived macrophages.

In the data presented here, both 5α-reduced glucocorticoid metabolites suppressed LPS-induced pro-inflammatory cytokine production, yet their efficacy compared to B was reduced, indicating that they do not retain the full anti-inflammatory potential of the parent glucocorticoid. To investigate possible cellular mechanisms behind these effects, protein levels of genes involved in inflammatory signalling pathways (Figure 5.9) were investigated in LPS-stimulated macrophages. Inflammation is initiated through the action of pro-inflammatory signals, including LPS, cytokines, viral factors and phorbol esters. Binding of these molecules to their cognate receptors initiates intra-cellular signalling cascades, ultimately resulting in the

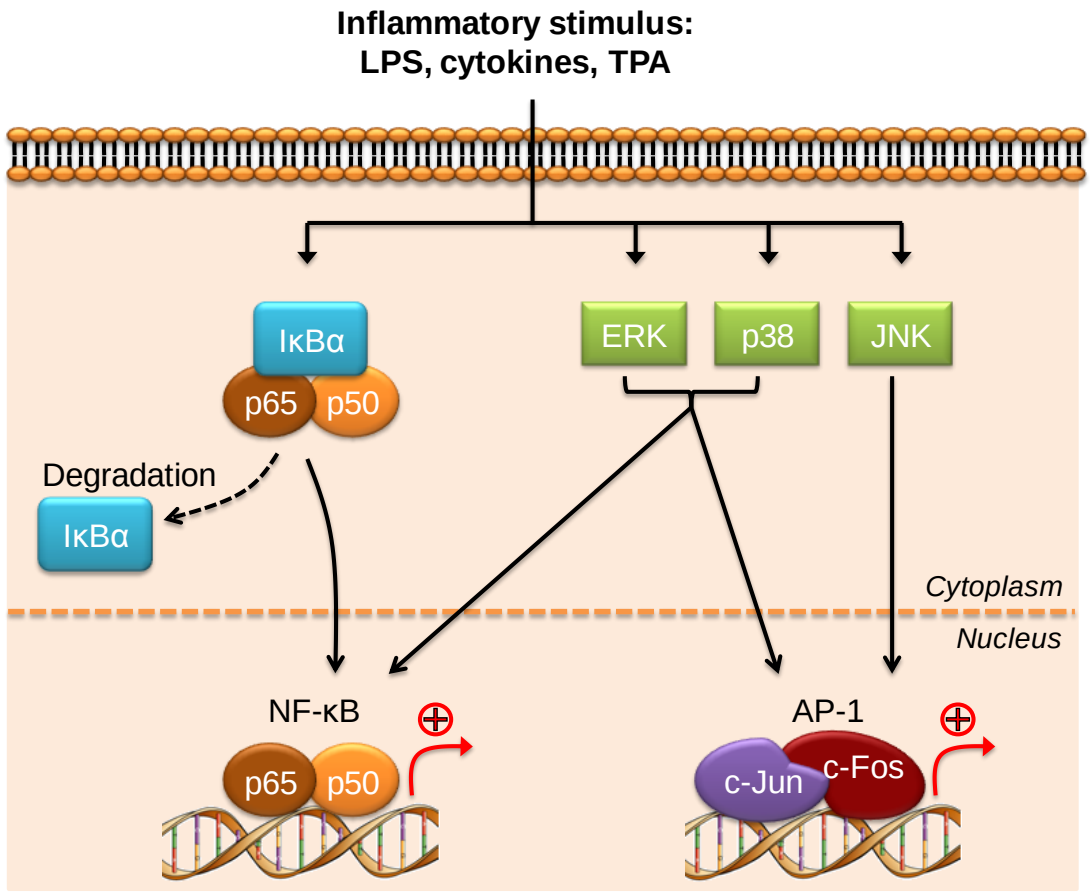

Figure 5.9 Overview of inflammatory signalling pathways. Stimulation via an inflammatory stimulus, whether it be lipopolysaccharide (LPS), pro-inflammatory cytokines such as TNFα, or a phorbol ester (TPA), results in the activation of multiple intra-cellular signalling cascades. The IκBα complex that holds NF-κB in the cytoplasm is phosphorylated and targeted for degradation, freeing NF-κB to translocate to the nucleus. Members of the MAPK family, including ERK, p38 and JNK are phosphorylated and thus activated by upstream kinases. This activation leads primarily to the activation of AP-1, but also, in the case of ERK and p38, the phosphorylation of NF-κB. The activation of both NF-κB and AP-1 leads to transcription of pro-inflammatory genes.

activation of the pro-inflammatory transcription factors NF-κB and AP-1 (Nissen and Yamamoto 2000; Hayden and Ghosh 2008; O'Neill 2008). However, such pathways involve multiple intermediary factors. One of these factors is IκBα, a protein that, in the resting state, is responsible for sequestering NF-κB within the cytoplasm (De Bosscher *et al.* 2003). However, upon inflammatory stimulation, an upstream kinase complex, namely IKK, phosphorylates IκBα, targeting it for degradation. This results in the release of NF-κB, enabling its translocation to the nucleus and subsequent transcriptional activity.

An important group of intermediary factors within inflammatory signalling pathways is the MAPK family of protein kinases, including JNK, p38 and ERK, all of which are activated through phosphorylation by upstream kinases. This family of MAPK proteins are particularly important in mediating AP-1 transcriptional activity, primarily through increasing expression of the AP-1 subunits c-Jun and c-Fos (Beyaert *et al.* 2002; Hazzalin and Mahadevan 2002; Johnson and Lapadat 2002). However, these kinases also have a role to play in the activation of NF-κB. In particular, both ERK and p38 act to phosphorylate the p65 subunit of NF-κB, ensuring the ability of the pro-inflammatory transcription factor to associate fully with transcriptional machinery and necessary coregulators (Goebeler *et al.* 2001). Naturally, inflammation is a highly regulated process and so the cell has several negative regulatory systems in place to ensure a balance in intracellular inflammation. With regard to the MAPK family, perhaps the most extensively studied negative regulator is MKP-1 (Camps *et al.* 2000; Salojin *et al.* 2006). This phosphatase is responsible for dephosphorylating the MAPK family of kinases, and thus inhibiting downstream activation of pro-inflammatory transcription factors. Given its role as a negative regulator, inflammatory stimuli induce protein expression of MKP-1, yet this is reported after approximately 60 minutes. However, both mRNA and protein expression immediately after stimulation (10 to 30 minutes), is known to be reduced (Cho and Kim 2009).

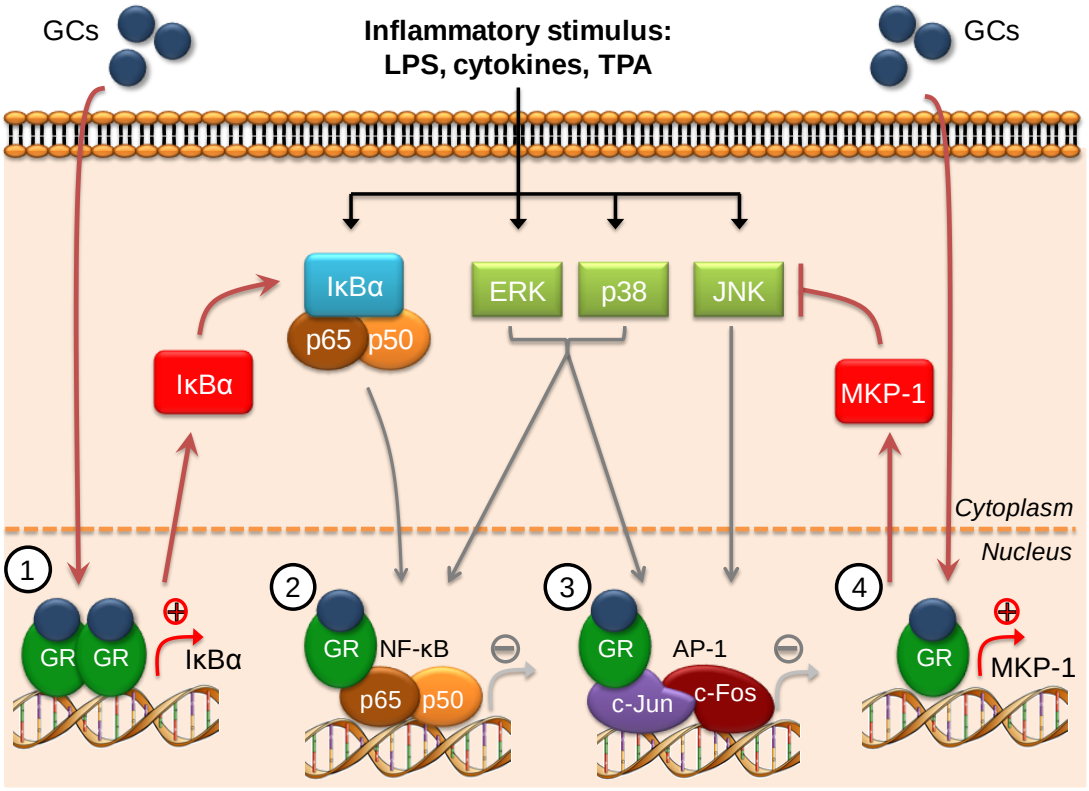

Figure 5.10 Intra-cellular glucocorticoid targets in inflammation. Glucocorticoids (GCs) are capable of acting at several sites to exert their anti-inflammatory effects. 1, glucocorticoid receptor (GR) homodimers induce expression of IκBα, which acts to sequester NF-κB in the cytoplasm, inhibiting downstream activation. 2+3, GR monomers tether to DNA-bound NF-κB and AP-1, preventing initiation of transcription. 4, GR monomer induces transcription of MKP-1, which serves to dephosphorylate the MAPK family of proteins, preventing activation and thus preventing further downstream signalling.

In order to explore the cellular mechanisms behind the effects of 5α-reduced glucocorticoid metabolites, it is important to understand first how glucocorticoids exert changes in gene expression (Figure 5.10). Glucocorticoids mediate their effects through ligation with the GR, a nuclear receptor with three functional domains, namely the N-terminal transactivation domain, activation-function 1 (AF1), the central DNA-binding domain (DBD) and the C-terminal ligand-binding domain (LBD) which also contains a second transactivation domain, AF2. Upon ligand binding, the GR dissociates from the multi-protein chaperone complex that maintains the GR in the cytoplasm, unmasking the nuclear localisation signal (NLS) and allowing translocation of the active GR to the nucleus. Within the nucleus, the anti-inflammatory effects of GR can be characterised primarily in two ways, transactivation of anti-inflammatory molecules and transrepression of pro-inflammatory molecules. In terms of transactivation, a number of genes are upregulated by glucocorticoids that have an anti-inflammatory role, including IκBα, MKP-1, IL-10 and lipocortin-1 (Clark 2007; Newton and Holden 2007). Transactivation primarily occurs through binding of GR homodimers to simple glucocorticoid-response elements (GREs) within target genes (Saklatvala 2002; Schacke *et al.* 2002). However, GR monomers may also play an important role through tethering mechanisms with other transcription factors including C/EBPα. Indeed, this is believed to be the mechanism behind glucocorticoid-inducible MKP-1 gene expression, with a study demonstrating that dexamethasone was still able to induce MKP-1 expression in macrophages with a mutated GR that prevented dimerisation (Abraham *et al.* 2006).

In our study, both 5α-reduced metabolites, along with B, significantly increased the amount of MKP-1 following LPS-mediated suppression. However, it is debatable whether the actions of these steroids serve specifically to induce protein expression of MKP-1, or prevent LPS-mediated degradation. It has previously been demonstrated in unstimulated RAW264.7 cells that dexamethasone treatment for 1 hour increases both mRNA and protein expression of MKP-1, an effect that was attenuated by co-incubation with the GR antagonist RU486 (Cho and Kim 2009).

This suggests that the increased amount of MKP-1 observed with 5α-reduced glucocorticoid metabolite treatment is a result of induction of protein expression rather than inhibited degradation. This upregulation also supports a role for MKP-1 in dephosphorylating the MAPK family (Kassel *et al.* 2001), given that treatment with both 5α-reduced glucocorticoid metabolites and B also resulted in a suppression of JNK and p38 phosphorylation. A similar situation to MKP-1 exists with IκBα, in which B and 5αDHB appear to increase protein expression. Again, it remains unclear whether this is due specifically to increased protein expression, or reduced IκBα degradation. To address these issues, it would be valuable to assess mRNA levels of both MKP-1 and IκBα to see if they follow the pattern shown in protein expression. Furthermore, the role of *de novo* protein synthesis could be addressed through co-incubation with protein synthesis inhibitors. This would not only provide evidence as to whether glucocorticoids actually induce protein expression, but in terms of MKP-1, it would reveal whether the upregulation of MKP-1 was essential for the suppression of JNK and p38 phosphorylation mediated by the steroids. In terms of IκBα, it was interesting that of the two 5α-reduced metabolites, only 5αDHB altered protein expression. A possible explanation for the lack of IκBα induction with 5αTHB may lie with an inability to induce dimerisation of GR. It has previously been shown that a dimerisation defective GR mutant is unable to enhance IκBα levels, indicating the importance of GR dimerisation in transactivation of IκBα (Heck *et al.* 1997). However, this mutant still retained the ability to repress NFκB activity, demonstrating that whilst IκBα plays a role in the anti-inflammatory effects of glucocorticoids, its overall importance remains debatable. Given that 5αTHB has been shown previously to have an inability to transactivate other glucocorticoid-responsive genes (Yang 2009), it would be interesting to look more closely at the effect that both 5α-reduced glucocorticoid metabolites have on GR dimerisation. Several studies have utilised the dimerisation-deficient GR (GR^{dim}) to assess whether the anti-inflammatory effects of GR ligands are dependent upon dimerisation (Reichardt *et al.* 1998; Tuckermann *et al.* 1999; Reichardt *et al.* 2001). Such a system would be extremely useful in determining whether 5αTHB loses any of its anti-inflammatory effects when incubated with this mutant GR compared to a wild type GR.

Despite the clear anti-inflammatory properties of proteins induced by glucocorticoids, their role in the overall anti-inflammatory response is controversial. The classical view is that the primary anti-inflammatory effects of glucocorticoids are mediated by transrepression of pro-inflammatory gene expression (De Bosscher and Haegeman 2009). Indeed, some studies have demonstrated that *de novo* protein synthesis plays little to no role in the ability of glucocorticoids to suppress expression of pro-inflammatory genes (De Bosscher *et al.* 1997; Wissink *et al.* 1998). Glucocorticoid-induced transrepression of pro-inflammatory gene expression is believed to mainly involve tethering of GR monomers with pro-inflammatory transcription factors such as NFκB and AP-1 (Beck *et al.* 2009). As such, direct DNA binding of GR is not required. However, the DBD still appears to be important in GR-mediated transrepression, as shown in a study using a GR with a mutation in the DBD (Bladh *et al.* 2005). This mutant GR was still able to repress AP-1 activity, but not NF-κB activity, indicating that not only is this region important even when DNA binding is not evident, but also that it is possible to discriminate between GR-mediated NF-κB and AP-1 repression. To investigate the effects of 5α-reduced metabolites specifically on pro-inflammatory transcription factor inhibition, we decided to utilise luciferase reporter plasmids with NF-κB of AP-1-binding sites. Initially, transfection of these plasmids in RAW264.7 cells was attempted. Although previous reports demonstrated transfection of RAW264.7 cells with FuGENE HD (Yamauchi *et al.* 2007; Shi and Kehrl 2008; Renga *et al.* 2009), in our hands, we could not achieve successful transfection, despite numerous optimising routines. Future work utilising other transfection reagents, such as Lipofectamine, which has been reported to induce transfection in these cells (Jiang and Pisetsky 2008), may yield more successful results.

In light of the inability to transfect RAW264.7 macrophages, an alternative cell line was used, namely HEK293 kidney cells, which had previously been utilised successfully with these plasmids (Bladh *et al.* 2005), and are a commonly transfected cell line. However, they lack the advantage of being an immune cell line. Whilst this

switch to a different cell line resulted in successful transfection, it also revealed some interesting results. Similar to B, 5αDHB suppressed TPA-stimulated transcriptional activity of both NF-κB and AP-1, indicating the ability of this metabolite to repress pro-inflammatory transcription factor activity. However, whilst 5αDHB retained the anti-inflammatory properties seen in macrophages, suppressing both NF-κB and AP-1 activation, 5αTHB actually increased activation of both transcription factors. This indicates that, in HEK293 cells, 5αTHB actually augments TPA-mediated inflammation, in direct contrast to the anti-inflammatory effects observed in macrophages. To further understand the different effects of 5αTHB in both cell types, the role of the GR in mediating these effects was investigated. In RAW264.7 cells, co-incubation with the GR antagonist inhibited the ability of both 5α-reduced metabolites to suppress LPS-induced pro-inflammatory cytokine release. This demonstrates that, in macrophages, anti-inflammatory effects of both 5αDHB and 5αTHB are mediated by GR. In HEK293 cells, actions mediated by GR were assessed by not transfecting cells with a GR plasmid. In the absence of GR, the inhibitory effects of B and 5αDHB on TPA-mediated transcriptional activity of NF-κB and AP-1 was ameliorated. Surprisingly, the absence of GR did not affect the ability of 5αTHB to augment NF-κB or AP-1-mediated transcription, indicating a GR-independent mechanism of action in HEK293 cells.

It has previously been shown that HEK293 cells express little to no endogenous GR (Bladh *et al.* 2005). Whilst it is possible that that residual endogenous GR is mediating the effects of 5αTHB, this seems highly unlikely given the apparent lack of effects induced by other steroids in the absence of co-transfected GR. Several alternative possibilities exist as to how 5αTHB exerts these effects independent of GR. Firstly, 5αTHB may be acting through another nuclear receptor within these cells. Aldosterone, the cognate ligand for the mineralocorticoid receptor (MR), has been shown to induce inflammation in heart and kidney cells (Gilbert and Brown 2010). As glucocorticoids are capable of binding and activating MR (Walker 2007), this provides a potential mechanism through which 5αTHB could further enhance inflammation. However, this again would appear unlikely given that characterisation

of HEK293 cells revealed an absence of endogenous MR (Ziera *et al.* 2009). Evidence also exists in neuronal cells that 5α-tetrahydro derivatives of both progesterone and cortisol are capable of acting via gamma-aminobutyric acid (GABA) receptors (Stromberg *et al.* 2005). These receptors are ligand-gated chloride ion channels that are found both in the central and peripheral nervous system, in both neuronal and non-neuronal cells. Whilst these receptors only appear to be present in cells involved with the nervous system, it highlights the potential of 5α-tetrahydro metabolites to bind to other receptors and modulate responses.

What has become evident from investigating the effects of 5α-reduced metabolites in both macrophages and kidney cells is the differences that exist between the two 5α-reduced glucocorticoid metabolites. It appears that, in macrophages, 5αDHB retains more of the anti-inflammatory effects of the parent glucocorticoid than 5αTHB, whilst in kidney cells, the effects of the two metabolites on inflammation differ. This highlights the cell-specific nature of these effects, as well as alluding to the role of the GR ligand in determining GR action. To understand why this may be the case, one must consider the fact that the GR belongs to a superfamily of nuclear receptors, and so previous work on other such receptors can provide valuable clues as to the nature of GR function. As mentioned above, all nuclear receptors within this family consist of the three domains described above, namely AF-1, DBD and LBD, and share many structural and functional features (Bledsoe *et al.* 2002; van der Laan and Meijer 2008). In terms of ligand-mediated effects, the LBD is perhaps the most interesting domain and serves several essential functions. It contains a binding pocket specific for the binding of the cognate ligand, as well as the second activation function (AF-2) (Bledsoe *et al.* 2002; McMaster and Ray 2007). The AF2 has been extensively studied in the androgen receptor (AR), and its contribution to receptor activity appears dependent upon both ligand- and cell-specific factors. In particular, AF-2 in known to recruit cofactors, including the p160 family of coactivators, upon ligand binding, thus enhancing AR activity. The recently published crystal structure of AR with bound T or 5αDHT has allowed for much greater insight into specific binding patterns and has shed valuable light on the differences in potency of these

two androgens (Askew *et al.* 2007). Naturally occurring mutations in AR have been shown to increase AR activity through enhanced coactivator recruitment to AF-2 (He *et al.* 2006) and, as such, the ability of AF-2 to recruit coactivators is now believed to be crucial to both the binding affinity and the activity of the AR.

T is a less potent androgen than 5αDHT, with a 10-fold higher concentration needed to achieve AR-mediated effects of 5αDHT, and dissociates 3x faster from AR than the latter (Askew *et al.* 2007). Structural data suggests that such differences can be attributed to differences within the H-bonding pattern between the ligand and the hydrophobic binding-pocket within the LBD of the receptor. Both T and 5αDHT have 19 carbons and differ only in that T has a $\Delta^{4,5}$ double bond in ring A. Unlike the saturated A-ring of 5αDHT, T has two fewer protons, ensuring a superior hydrophilic property relative to 5αDHT and, therefore, greater H-bonding potential. The crucial bond in this pattern appears to be between 3-keto O on the steroid and Arg752, a conserved helix 5 residue on the receptor. The $\Delta^{4,5}$ double bond in T has a more planar angle than 5αDHT, ensuring more favourable H-bonding (Askew *et al.* 2007). One might hypothesise that greater bonding would result in greater affinity. However, this does not appear to be the case here. Indeed, the 'counterintuitive hypothesis' states that greater H-bonding is detrimental to agonist activity. This is believed to be due to the introduction by T of an unfavourable hydrophilic character within the hydrophobic binding pocket (Askew *et al.* 2007). This is evidenced by the network of H-bonds through structural water HOH1 between the steroid and key residues within the AF2 domain. The planar structure of T would appear to allow T to accept a bridged H-bond with Met745. In contrast, the lack of a $\Delta^{4,5}$ double bond in 5αDHT appears to weaken or eliminate such a bridged H-bond. This Met745 residue in the AF2 domain lies directly above the Δ4,5 double bond of the steroid, and projects towards Leu712, a proximal residue within AF2. This residue is crucial in mediating hydrophobic contacts with residues within cofactor molecules. A mutation in this residue has been shown to cause androgen insensitivity without altering androgen binding affinity, demonstrating that the recruitment of cofactors is paramount to the activity of this receptor (Ghali *et al.* 2003). 5αDHT has been

suggested to impart greater structural integrity to Leu712, permitting greater cofactor binding and thus greater AR activity (Askew *et al.* 2007).

These findings with AR show that A-ring chemistry can have direct effects on cofactor recruitment. However, ligand-mediated activities only form part of the picture, as the presence of specific cofactors can also determine receptor activity, possibly explaining the cell-specific effects of steroids. Indeed, this has been shown when investigating the estrogen receptor (ER) antagonists tamoxifen and raloxifene (Shang and Brown 2002). Both of these antagonists were shown to recruit corepressors to target genes in mammary cells, thus repressing gene transcription. However, in endometrial cells, tamoxifen acts as an ER agonist, inducing gene transcription through recruitment of coactivators. This demonstrates the ability of a steroid receptor ligand to act as either an agonist or antagonist depending on the cellular environment. This may provide an explanation for the differential effects of 5αTHB on inflammation between macrophages and kidney cells. The reasons behind the ability of different ligands to recruit either coactivators or corepressors to their cognate receptor appear to be related to the structural changes that occur upon ligand binding. This has been investigated for the binding of the GR agonist dexamethasone and the GR antagonist mifepristone (Schoch *et al.* 2010) (Figure 5.11). Binding of dexamethasone to GR places helix 11 in an 'agonist' conformation, favouring binding of the transcriptional intermediary factor-2 (TIF2) coactivator (TIF2 is the human homologue of murine GRIP1) (Figure 5.11a). The antagonist RU486, which binds to the GR-LBD in the same location and orientation as dexamethasone, induces a distinctly different conformation within the GR (Figure 5.11b). Helix 11 is displaced from its agonist position, creating a cofactor binding site that has a bigger volume than the site forTIF2. This results in the inability to form charge interactions between Glu755 from helix 11 and the N-terminus of the TIF2-interacting helix. However, the nuclear receptor corepressor (NCoR) is now able to bind, forming interactions via its C-terminus with Lys759 of the GR (Schoch *et al.* 2010). This work demonstrates that agonist or antagonist activity of the GR is dependent upon

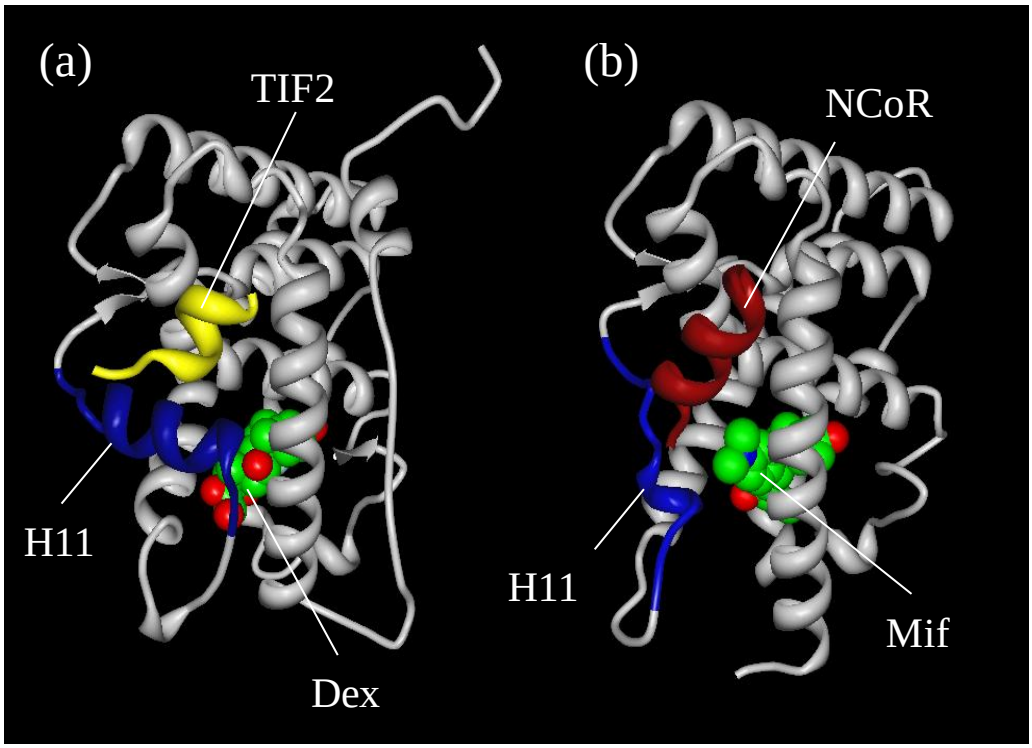

Figure 5.11 Conformational changes induced in helix 11 of the GR by different ligands. In (a), dexamethasone (Dex) binding results in a conformational change in the position of helix 11 (H11) to the agonist position. This allows the recruitment of the transcriptional intermediary protein-2 (TIF2) coactivator. In (b), RU486 (mifepristone, Mif) displaces helix 11 from the agonist position, enlarging the coregulator binding site and thus permitting the binding of the nuclear corepressor (NCoR). Figures were generated using the RCSB Protein Workshop software.

the ability to recruit coactivators or corepressors and similar mechanisms may underpin the difference in actions between 5αDHB and 5αTHB.

The effects of 5α-reduced glucocorticoid metabolites most likely involve the recruitment of such cofactors, however, this has yet to be investigated. What remains perplexing is the ability of 5αTHB to suppress inflammation in one cell type, but induce it in another. Furthermore, it remains to be fully established whether the effects of 5αTHB in the kidney cells where it enhanced inflammation is mediated through GR. If 5αTHB does indeed act via GR in these cells, what appears a likely scenario is that it recruits a different set of cofactors to both B and 5αDHB, and whilst these cofactors are present in macrophages, permitting the suppression of inflammation, they are absent in kidney cells. As shown above, ligand binding determines the ability of the receptor to recruit such cofactors. Whilst this has been shown for GR binding with dexamethasone (Schoch *et al.* 2010), no studies exist on 5α-reduced metabolite binding. Of course, the cell-specific effects of 5αTHB, along with the difference in effects between the two metabolites highlight potential *in vivo* implications for the use of 5α-reduced metabolites of glucocorticoids. Given their ability to cause cell-specific effects, it raises the question as to whether they would provide desirable effects if administered as an anti-inflammatory agent. Furthermore, the fact that these steroids are metabolites leads to the issue of whether 5αDHB would be metabolised to 5αTHB in the body, thus negating any potential anti-inflammatory benefits. To investigate this, the expression of A-ring enzymes were investigated in RAW264.7 macrophages. In these cells, it was found that only 5α-reductase type 1, the enzyme responsible for reducing the parent glucocorticoid to its 5α-dihydro metabolite, and not 3αHSD, was present. This indicates that the effects observed following administration of either 5αDHB or 5αTHB were due specifically to that steroid, and not as a result of it being further metabolised within the cell. However, within the body, distribution of these enzymes is tissue-specific and so it remains a distinct possibility that when administered *in vivo*, these steroids would be metabolised, particularly within the liver.

In summary, 5α-reduced glucocorticoid metabolites have anti-inflammatory effects in macrophages, but, at equilmolar concentrations, are less effective than the parent glucocorticoid. The ability to suppress pro-inflammatory cytokine production in macrophages is mediated through GR. At the molecular level, the anti-inflammatory effects appear to be mediated, in part, through inhibition of inflammatory kinase phosphorylation and activation. For the parent glucocorticoid, this inhibition correlates with an increase in the levels of MKP-1, a phosphatase responsible for dephosphorylating these inflammatory kinases. This may also be the case for the 5α-reduced glucocorticoid metabolites, however, the evidence here suggests not. The effects of 5αTHB appear to be cell-specific, given the differential effects on inflammation in macrophages and kidney cells. More work is necessary in order to establish the mechanism behind such cell specific effects, possibly utilising chromatin immunoprecipitation (ChIP) to investigate the association of cofactors with 5αTHB-bound GR, as well as utilising cofactor-binding inhibitors to determine if the effects of 5αDHB are dependent upon cofactor associations. It would also be exciting to determine whether these metabolites are capable of inducing GR dimerisation or whether the effects are mediated by GR monomers. However, this work provides new mechanistic insights into the actions of 5α-reduced glucocorticoid metabolites *in vitro*. In terms of the dysregulation of A-ring metabolism observed in obesity (Andrew *et al.* 1998; Livingstone *et al.* 2005), it is interesting to hypothesise the consequence of increased hepatic 5αR1 expression. The parent glucocorticoid acts in the liver to increase gluconeogenesis, as well as affecting cycling of glycogen, such that the body is primed to be responsive (Sugiyama *et al.* 1998; van Schaftingen and Gerin 2002; Dostert and Heinzel 2004; Macfarlane *et al.* 2008). Whilst this is necessary in the setting of starvation, such action in the setting of obesity is detrimental. The increased expression of 5αR1 in the liver is believed to be an adaptation to obesity, protecting the liver from the harmful metabolic actions of glucocorticoids by inactivating them. Although an attractive hypothesis, this implies that the 5α-reduced glucocorticoid metabolites formed are inert, which, as demonstrated in previous studies and in this thesis, is untrue. Whilst the metabolic effects of 5αDHB are yet to be fully established, 5αTHB has reduced metabolic actions compared to the parent glucocorticoid (Yang

2009). In terms of the immunomodulatory effects of glucocorticoids, both 5α-reduced metabolites exert clear anti-inflammatory effects, although the effects appear to be cell-specific for the 5α-tetrahydro form. This has important consequences as hepatic inflammation is clearly evident in the setting of obesity. Therefore, it suggests that the liver is not merely 'disposing' of local glucocorticoid in an attempt to prevent their metabolic action, but actively producing a form of the glucocorticoid that retains the anti-inflammatory action of the parent and may serve to suppress the local inflammation.

Chapter 6

Investigation into the anti-inflammatory properties of 5α-reduced glucocorticoids *in vivo*

6.1 Introduction

As outlined in Chapter 5, both 5α-reduced metabolites of corticosterone are capable of exerting anti-inflammatory effects *in vitro.* In murine macrophages, both 5αDHB and 5αTHB suppressed release of the pro-inflammatory cytokines TNFα and IL-6 from LPS-stimulated macrophages. This suppression in macrophages appears to be mediated through inhibition of both NF-κB and AP-1 activation, as shown by changes in the both total protein and phosphorylation states of several molecules involved in these inflammatory pathways. However, in a distinctly different cell type, namely kidney cells (HEK293), only 5αDHB was capable of suppressing inflammatory signalling, with 5αTHB actually increasing the activation of pro-inflammatory transcription factors. Such a discovery poses an intriguing question as to whether there are tissue specific effects of these steroids which may influence their spectrum of anti-inflammatory effects. There is a need for alternative glucocorticoid drugs due to the detrimental side effects of chronic conventional glucocorticoid treatment for inflammatory conditions, including osteoporosis and development of type 2 diabetes (Saklatvala 2002; Schacke *et al.* 2002; Wei *et al.* 2004). Previous work in mice has indicated that, in contrast to corticosterone, chronic exposure to 5αTHB does not affect body weight, blood pressure or insulin sensitivity (Yang 2009), although 5αDHB has yet to be tested. The potential anti-inflammatory effects of both 5αDHB and 5αTHB remain to be investigated *in vivo*.

Hypothesis

The hypothesis of this chapter is that 5α-reduced metabolites of corticosterone suppress inflammation in an *in vivo* setting.

Aims

The aims of this chapter were to investigate:

1) Whether 5α-reduced metabolites of corticosterone suppress inflammation associated with thioglycollate-induced peritonitis.

2) Whether the effects of 5α-reduced corticosterone metabolites in the above model are comparable in terms of efficacy to the parent glucocorticoid corticosterone.

6.2 Materials and methods

6.2.1 Experimental outline

Steroid injections were administered 2 hours prior to the induction of peritonitis with thioglycollate, with tail-nick blood immediately prior to thioglycollate administration. Four hours after induction of peritonitis, animals were sacrificed by decapitation, trunk blood taken, and peritoneal lavages performed.

6.2.2 Animal Maintenance

Adult male C57Bl/6 mice (12 weeks) were maintained as described (2.3.1).

6.2.3 Thioglycollate-induced peritonitis

Thioglycollate-induced peritonitis was induced as described (2.4.3).

6.2.4 Preparation of steroid solutions

To assess dose response relationships, corticosterone (2.5, 7.5, 25, 75, 250 mg/ml) was prepared dissolved in vehicle of DMSO. For comparison of corticosterone with 5α-reduced metabolites, solutions containing corticosterone, 5αDHB or 5αTHB (25, 75 and 250 mg/ml) were prepared dissolved in a vehicle of hydro-propyl-β-cyclodextrin in DMSO (5% w/v) immediately prior to use.

6.2.5 Dose response to corticosterone to suppress inflammation

Two hours prior to induction of peritonitis (09:00), corticosterone (2.5, 7.5, 25, 75 and 250mg/ml) or vehicle was administered by subcutaneous injection (20µl).

6.2.6 Comparison of the efficacy of steroids to suppress inflammation

Two hours prior to induction of peritonitis (09:00h), corticosterone, 5αDHB, 5αTHB (25, 75, 250mg/ml) or vehicle were administered by subcutaneous injection (20µl).

6.2.7 Terminal procedures

Animals were sacrificed as described (2.4.4)

6.2.8 Peritoneal lavage

Peritoneal lavages were performed as described (2.4.5).

6.2.9 Quantification of total cell infiltration

Cells within peritoneal lavage fluid were primarily quantified by NucleoCounter (6.2.9.1). However, in the absence of NucleoCassettes, cells were quantified by haemocytometer (6.2.9.2).

6.2.9.1 Quantification of cell infiltration by NucleoCounter

Peritoneal lavage samples were subjected to centrifugation (1000g, 5min, RT), with the cell pellet resuspended in BSA (Fraction V, 0.1% w/v, 0.5ml). The cell suspension was diluted (1 in 10 in 0.1% w/v BSA) and the total number of inflammatory cells was determined using a NucleoCassette and a NucleoCounter NC-100 (ChemoMetec, Denmark). The two reagents provided (Reagent A-100 and Reagent B) were each added (50µl) into the suspension and mixed. Reagent A-100 (pH 1.25) is a lysis buffer that disaggregated cell clusters and disrupted cell membranes. Reagent B was a stabilizing buffer that raised the pH value and allowed more efficient staining of DNA in the NucleoCassette. The fluorescent dye propidium iodide is immobilized within the flow channels of the NucleoCassette. Upon addition of sample mixture (50µl), the propidium iodide is dissolved and the

DNA stained. The loaded NucleoCassette was then placed in the NucleoCounter and the total cell number was generated automatically by NucleoView Software (ChemoMetec, Denmark).

6.2.9.2 Quantification of cell infiltration by haemocytometer

A coverslip was firmly attached on top of the chamber of the haemocytometer, with lavage fluid (10µl) added to both sides of the chamber. Cells were counted in the four corner quadrants of each side of the chamber. For each side, the total number was divided by four, and the average of both sides equated to the number of cells per mm^2. This value was multiplied by 10^4 to give the number of cells per ml of the cell suspension.

6.2.10 Quantification of inflammatory cells by fluorescence activated cell sorting (FACS) analysis

6.2.10.1 Preparation of antibody mixes

Alexa Fluor 647 conjugated monoclonal rat-anti-mouse 7/4 (IgG2a, AbD Serotec, Oxfordshire, UK), fluorescein isothiocyanate (FITC) conjugated monoclonal rat-anti-mouse Ly-6G (IgG2a, BioLegend, CA, USA), phycoerythrin (PE) conjugated rat-anti-mouse monoclonal F4/80 (IgG2a, Invitrogen, Paisley, UK) and tandem Peridinin Chlorophyll Protein Complex/Cy5.5 (PerCP/Cy5.5) conjugated monoclonal rat-anti-mouse CD11b (Biolegend, CA, USA) were diluted (1/100) in mouse serum (10% v/v in PBS). Single stain controls consisting of each of the antibodies above separately were also prepared, as were isotype controls for each antibody (purchased from same company as fluorescent form of antibody).

6.2.10.2 Preparation of peritoneal cells

Peritoneal lavage samples were mixed thoroughly and transferred to chilled FACS tubes (BD Falcon, Oxfordshire, UK) (300µl). Mouse serum (30µl) was added to block non-specific binding sites and incubated (10 min, 4°C). Appropriate antibody mixes were added to each sample (50µl), followed by mixing and incubation in the dark (30 min, 4°C). Peritoneal cells were washed with PBS (1ml) and subjected to centrifugation (300g, 5 min, 4°C) to form a pellet. The supernatant was removed and cells fixed with formalin (10% v/v in PBS, 100µl) until analysis.

6.2.10.3 FACS analysis

Analysis was performed using the LSRFortessa Cell Analyser (BD Biosciences, Oxfordshire, UK). Specific and non-specific fluorescence was determined by processing $\geq$ 5000 cells per sample. Data analysis was performed using FlowJo software (Treestar, OR, USA). Myeloid cells were identified as CD11b positive cells and were further distinguished based upon staining for Ly6G, 7/4 and F4/80 as described (Henderson *et al.* 2003). Ly6G is a marker for neutrophils that is not expressed on monocytes or macrophages (Cash *et al.* 2009), whilst 7/4 is a marker for recently generated inflammatory monocytes, as well as neutrophils (Cash *et al.* 2009; Rosas *et al.* 2010). F4/80 serves as a marker for macrophage differentiation, allowing distinction between monocytes and macrophages (Cash *et al.* 2009; Rosas *et al.* 2010). A combination of these markers was used to identify distinct populations of neutrophils and inflammatory monocytes. Neutrophils were characterised by high expression of 7/4, and are distinguished from monocytes and macrophages due to their high expression of Ly6G (Figure 6.1). Analysis of $7/4^{hi}$, $Ly6G^{lo}$ cells revealed low expression of the F4/80, indicating these cells were newly recruited inflammatory monocytes.

6.2.11 Quantification of cytokines within peritoneum by ELISA

Cytokine levels within the peritoneal lavage fluid were quantified by ELISA as described for cell media (2.6.7).

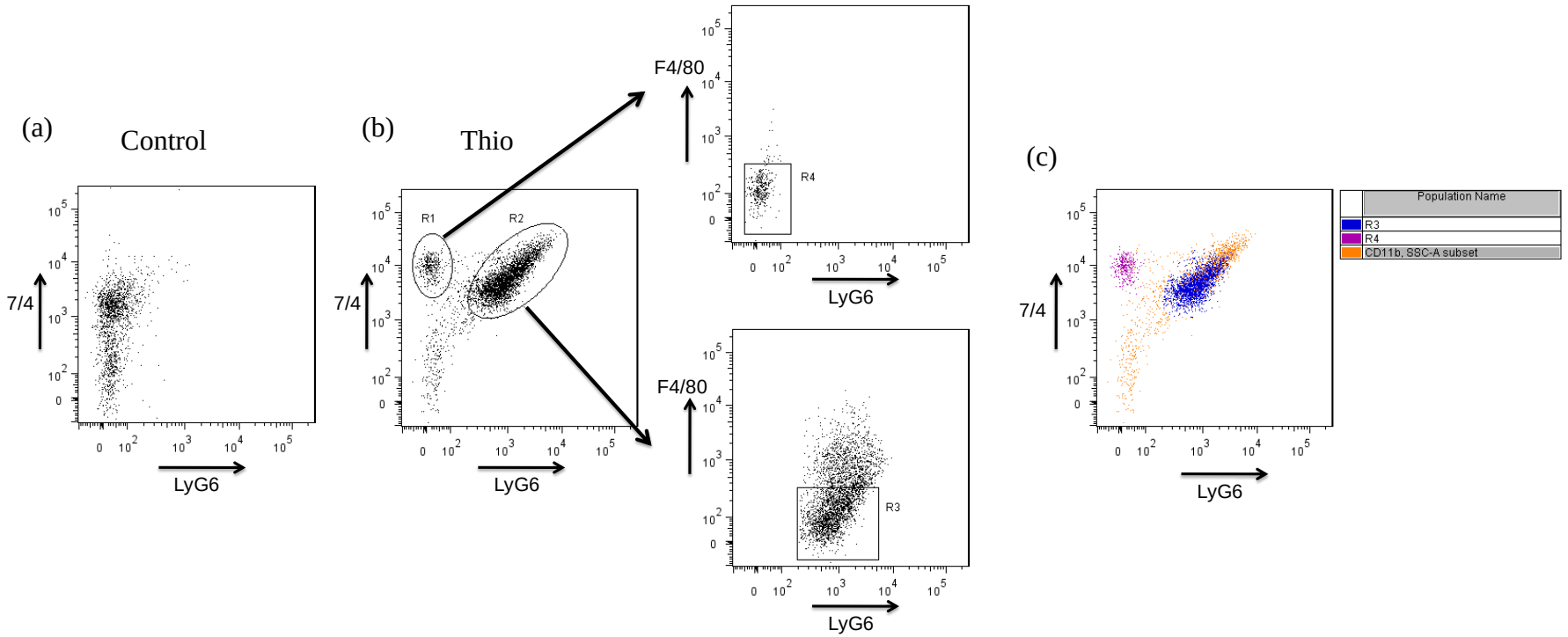

Figure 6.1 Flow cytometric analysis of myeloid cells. Peritoneal myeloid were analysed by staining with 7/4 and Ly6G 4 hours after PBS control (a) or thioglycollate injection (Thio) (b). Appearance of Ly6G^{hi}, 7/4^{hi} cells (R2) in thioglycollate treated lavage (b), which contains two distinct populations of cells, of which the F4/80^{lo} population consists of neutrophils. The Ly6G^{lo}, 7/4^{hi} population (R1) consists primarily of F4/80^{lo} cells, which are inflammatory monocytes. The location of these cells within the scatter of Ly6G and 7/4 is shown in (c), with neutrophils (R3) in blue, inflammatory monocytes (R4) in purple, and remaining myeloid cells in orange.

6.2.12 Quantification of plasma corticosterone by RIA

Plasma corticosterone levels were quantified by RIA as described (2.6.5).

6.2.13 Statistics

Data are presented as mean ± SEM and were analysed by One-way Analysis of Variance (ANOVA) with described post-hoc tests as appropriate.

6.3 Results

6.3.1 Dose response to corticosterone to suppress inflammation

Plasma corticosterone concentrations increased following administration of the steroid in a dose responsive manner (Figure 6.2). Peak physiological levels are highlighted, demonstrating that the concentrations reached following corticosterone administration are pharmacological. Compared to control injection of PBS, thioglycollate injection increased the number of cells infiltrating the peritoneum. The two highest doses of B (75 and 250 mg/ml) significantly suppressed peritoneal cell infiltration (Figure 6.3).

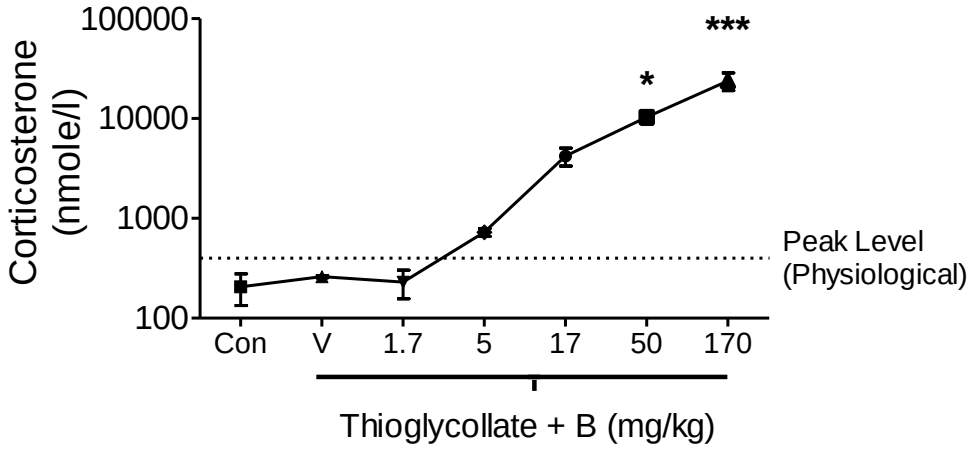

Figure 6.2 Plasma corticosterone increased in response to corticosterone injection. Corticosterone (B) levels in plasma 2 hours post-corticosterone administration were quantified by RIA. Circulating levels elevated in accordance with increasing dose of corticosterone. Data are mean ± SEM for n=3-4 per group. Con = Control (PBS). Comparisons were by one-way ANOVA with Dunnett's post-hoc tests. *P<0.05 vs Vehicle (V, DMSO).

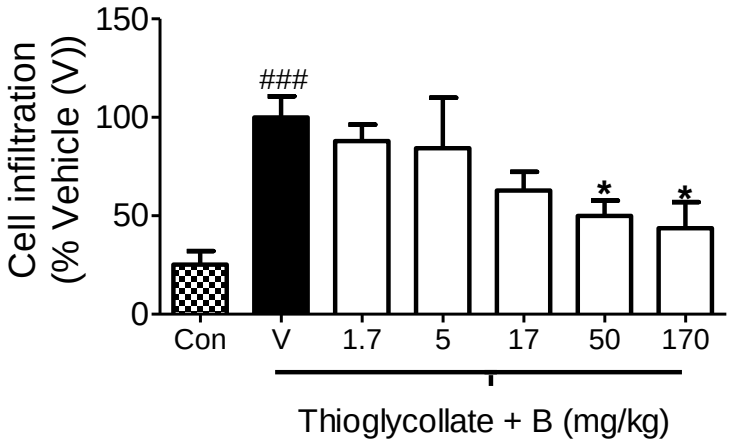

Figure 6.3 Peritoneal infiltration in response to corticosterone. Peritoneal cell infiltration following thioglycollate challenge quantified by NucleoCounter, with effect of corticosterone (B) pre-treatment expressed as percentage of response to Vehicle (V). Cell infiltration was suppressed with increasing concentration of B. Data are mean ± SEM for n=3-4 per group. Comparisons were by one-way ANOVA with Dunnett's post-hoc tests. ###P<0.001 vs Con (Control, PBS, hatched bar); *P<0.05 vs V (Vehicle, DMSO, black bar).

6.3.2 Suppression of thioglycollate-induced peritonitis by 5α-reduced glucocorticoid metabolites

6.3.2.1 Suppression of cell infiltration into peritoneum

The parent glucocorticoid B suppressed cell infiltration into the peritoneum in a dose-dependent manner. Both 5αDHB and 5αTHB also suppressed the influx of cells into the peritoneum (Figure 6.4). At the highest dose tested, there were no significant differences in terms of efficacy between the steroids.

6.3.2.2 Identification of cell populations within peritoneal infiltrate

In order to establish whether this reduction in the number of cells in the peritoneum was universal for all types of inflammatory cells, or if a specific subset of cells were affected, peritoneal cells from these mice was analysed by FACS. In mice given a control injection of PBS, 1.1 ± 0.2% of the cells were neutrophils and 0.2 ± 0.05% were inflammatory monocytes. Thioglycollate injection induced significant infiltration of both neutrophils and monocytes. The predominant population was neutrophils, which made up 59.7 ± 1.5% of cells present within the peritoneal lavage following thioglycollate injection, whilst inflammatory monocytes comprised 3.7 ± 0.5% of infiltrating cells. Neutrophil numbers were reduced to a similar extent by all doses of B (Figure 6.5a), whilst inflammatory monocyte infiltration was suppressed in a dose-dependent manner by B (Figure 6.5b). Both 5αDHB and 5αTHB suppressed neutrophil infiltration to a similar extent to B (Figure 6.5a). Inflammatory monocyte infiltration was suppressed by 5αDHB and 5α THB, both of which were less efficacious than B at the highest dose (Figure 6.5b).

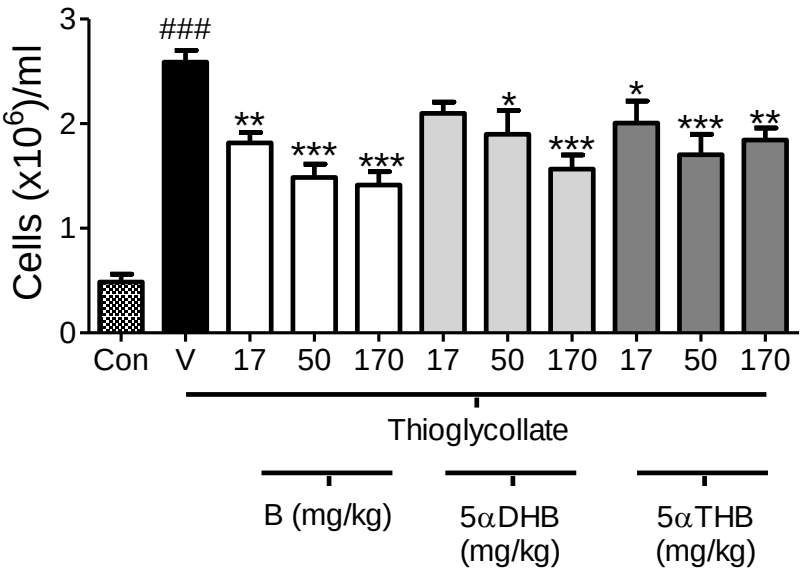

Figure 6.4 5α-Reduced metabolites reduce cell infiltration in peritoneum. Total cell influx into the peritoneal cavity 4 hours after induction of peritoneal inflammation by thioglycollate injection was quantified using a haemocytometer. Mice were pre-treated with increasing doses of corticosterone (B), 5α-dihydrocorticosterone (5αDHB) or 5α-tetrahydrocorticosterone (5αTHB) for 2 hours prior to induction of inflammation. Both 5α-reduced metabolites suppressed infiltration of cells into the peritoneum along with the parent glucocorticoid B. V = steroid vehicle (5% β-cyclodextrin in DMSO). Data are mean ± SEM for n=6 per group. Comparisons were by one-way ANOVA with Dunnett's post-hoc tests. ###P<0.001 vs Con (Control, PBS, hatched bar); *P<0.05, **P<0.01, ***P<0.001 vs V (Vehicle, 5% β-cyclodextrin in DMSO, black bar).

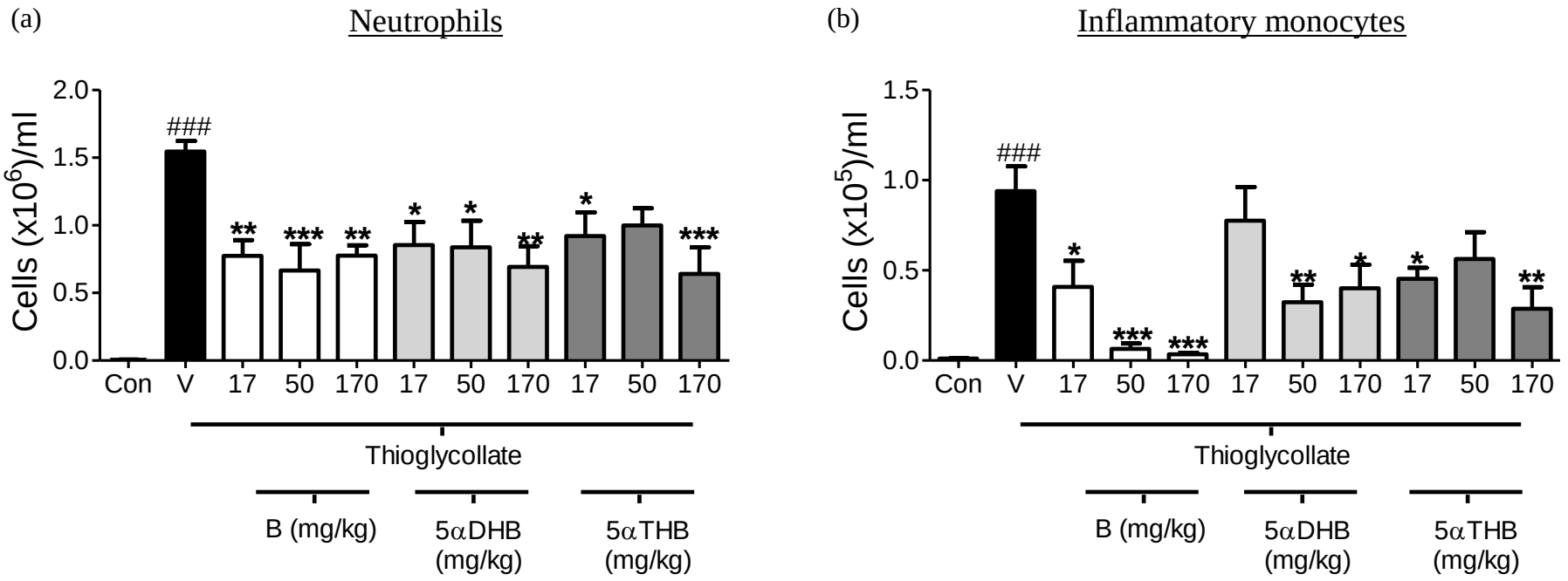

Figure 6.5 Suppression of peritoneal neutrophil and inflammatory monocyte infiltration. The population of cells entering the peritoneum 4 hours after thioglycollate injection was assessed by flow cytometry. Both 5α-dihydrocorticosterone (5αDHB) and 5α-tetrahydrocorticosterone (5αTHB) suppressed the influx of neutrophils into the peritoneum, as did the parent glucocorticoid B (a). B suppressed the increased influx of inflammatory monocytes into the peritoneum in a dose-dependent manner. Whilst 5αDHB and 5αTHB also reduced the number of inflammatory monocytes, they were less efficacious than the parent glucocorticoid (b). Data are mean ± SEM for n=6 per group. Comparisons were by one-way ANOVA with Dunnett's post-hoc tests. ###P<0.001 vs Con (Control, PBS, hatched bar); *P<0.05, **P<0.01, ***P<0.001 vs V (Vehicle, 5% β-cyclodextrin in DMSO, black bar).

6.3.2.3 Peritoneal cytokine and chemokine levels

IL-6 levels within the peritoneum were increased following thioglycollate stimulation, whilst treatment with B suppressed this rise (Figure 6.6a). 5αDHB also reduced IL-6 levels, but was less efficacious than B, only achieving suppression at the highest dose tested. Interestingly, 5αTHB suppressed IL-6 levels at the lowest dose tested, but failed to suppress at higher doses (Figure 6.6a). The results for MCP-1 are similar, with B reducing peritoneal levels. Again, 5αDHB suppressed levels, but only at the highest dose, whilst 5αTHB had no significant effect on MCP-1 levels (Figure 6.6b).

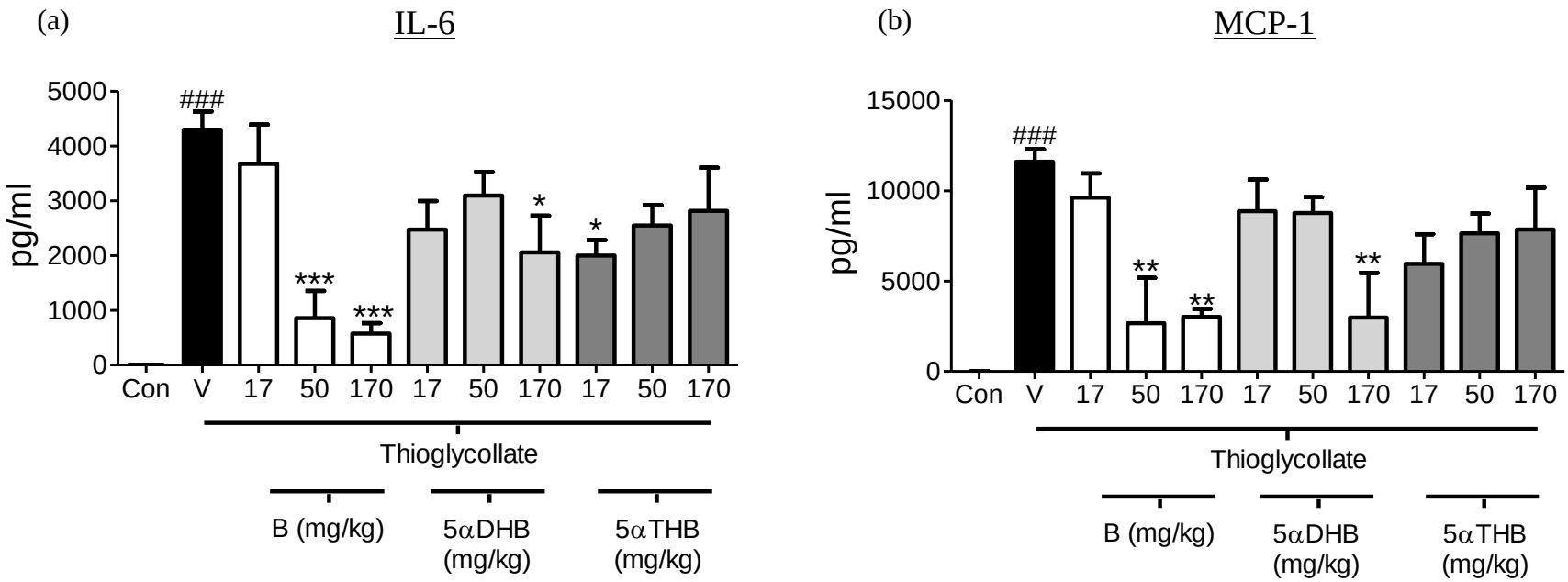

Figure 6.6 Peritoneal cytokine and chemokine levels. IL-6 (a) and MCP-1 (b) levels were quantified in peritoneal lavage fluid 4 hours after thioglycollate injection by ELISA. Corticosterone (B) suppressed release of IL-6 to a greater extent than the both 5α-dihydrocorticosterone (5αDHB) and 5α-tetrahydrocorticosterone (5αTHB) (a). Similar to IL-6, MCP-1 levels were suppressed by several doses of B, yet only the highest dose of 5αDHB had a suppressive effect. Data are mean ± SEM for n=6 per group. Comparisons were by one-way ANOVA with Dunnett's post-hoc tests. ###P<0.001 vs Con (Control, PBS, hatched bar); *P<0.05, **P<0.01, ***P<0.001 vs V (Vehicle, 5% β-cyclodextrin in DMSO, black bar).

6.4 Discussion

In this chapter, thioglycollate-induced peritonitis was used as an inflammatory model in which to assess the potential anti-inflammatory effects of 5α-reduced glucocorticoid metabolites. The total number of cells infiltrating the peritoneum following thioglycollate challenge was significantly reduced with both 5αDHB and 5αTHB pre-treatment, similar to that seen with the parent glucocorticoid B. Analysis of these cells revealed that neutrophil numbers were reduced to a similar extent following pre-treatment with all doses of steroids. Infiltration of inflammatory monocytes was also altered by steroid pre-treatment, with B having the most significant suppressive effect. Although less efficacious than B, both 5αDHB and 5αTHB also reduced inflammatory monocyte numbers. B suppressed the levels of both IL-6 and MCP-1 within the peritoneum, but the effects of 5α-reduced glucocorticoid metabolites were less pronounced, with only the highest dose of 5αDHB suppressing IL-6 and MCP-1 levels. Interestingly, the lowest dose of 5αTHB suppressed IL-6 levels, whilst the higher doses had no significant effect. 5αTHB did not alter MCP-1 levels.

Inflammation is the response of tissues to injury and infection. Acute inflammation is normally resolved within a matter of days. However, some inflammatory responses, classified as chronic inflammation, persist over an extended period of weeks and even years, leading to irreversible tissue remodelling and damage (Nathan 2002; Medzhitov 2008). In terms of acute inflammation, neutrophils are rapidly recruited to the site of infection or injury and form the basis of infiltrating leukocytes. The classical view has been that later in the inflammatory response, leukocytes of the monocyte and macrophage lineage are recruited, thus becoming the dominant leukocyte population at the site of infection. This switch in profile is thought to play a critical role in the inflammatory response, as macrophages phagocytose leukocytes that are undergoing apoptosis, preventing the release of intracellular pro-inflammatory molecules and enabling the resolution of inflammation (Medzhitov 2008). Glucocorticoids are well established anti-inflammatory steroids, and

pharmacological doses have multiple anti-inflammatory effects on numerous cell types (Saklatvala 2002). In terms of neutrophils, glucocorticoids act to inhibit their chemotactic activity, preventing their migration to the site of infection (Pitzalis *et al.* 2002). The release of pro-inflammatory cytokines and chemokines from macrophages is also suppressed by glucocorticoids, as demonstrated in Chapter 5, as well as in several other reports (Reichardt *et al.* 2001; Tuckermann *et al.* 2007). Furthermore, glucocorticoids have been shown to promote macrophage phagocytosis of apoptotic neutrophils, potentially promoting the resolution of inflammation (Heasman *et al.* 2003).

Injection of pathogens into the peritoneal cavity is widely used as a murine model to study acute inflammation. These models have allowed insights into the specific mechanism of inflammatory responses, including the cell types involved and the time course over which the inflammatory response occurs. Administration of thioglycollate into the mouse peritoneum leads to an acute inflammatory response that peaks within hours (4-8h) and resolves within days (3-4 days) (Melnicoff *et al.* 1989; Henderson *et al.* 2003; Chen *et al.* 2008). The peak response is characterised as the time-point at which the greatest number of neutrophils have been recruited to the peritoneal cavity, and so for this study, we chose to assess cell infiltration into the peritoneum 4h after thioglycollate challenge. Of further importance was the selection of a model which was glucocorticoid-responsive. A previous study has shown that treatment with the synthetic glucocorticoid dexamethasone suppresses cell infiltration following intra-peritoneal injection of thioglycollate in C57Bl/6 mice (Montesinos *et al.* 2006), demonstrating that this is an appropriate choice of model to study the potential anti-inflammatory effects of 5α-reduced glucocorticoid metabolites.

Whilst dexamethasone has been shown to suppress inflammation in thioglycollate-induced peritonitis, previous data does not exist on the anti-inflammatory ability of corticosterone. Therefore, to establish a comparable working model of

glucocorticoid-induced suppression of inflammation, the effect of B was explored in a 4 hour, acute model of thioglycollate-induced peritonitis. The dose of dexamethasone previously described to suppress cell infiltration equates to approximately 2.3 mg/ml. Given that dexamethasone is approximately 30x more potent than endogenous glucocorticoids, a corticosterone dose in the region of 69 mg/ml would be expected to have a similar effect. Total peritoneal cell infiltrate was suppressed with increasing concentration of B, (IC50 = 73.9 mg/ml) demonstrating the anti-inflammatory effects of corticosterone are in accordance with that seen with dexamethasone. An increase in the circulating levels of B was observed in line with the increasing doses administered. Physiological levels of circulating B in mice peaks at approximately 260 nmoles/L, however this rises to approximately 400 nmoles/L under conditions of stress, such as acute restraint (Carter *et al.* 2009). Given that only the top two doses suppressed cell infiltration, it is evident that pharmacological doses of corticosterone are necessary to achieve anti-inflammatory effects.

Having demonstrated in Chapter 5 that the 5α-reduced glucocorticoid metabolites retain similar anti-inflammatory properties to B *in vitro*, it was important to establish whether or not these properties were displayed *in vivo*. The three highest doses used for the corticosterone (B) dose response were chosen to test the effects of the 5α-reduced metabolites. Following thioglycollate injection, B reduced cell infiltration into the peritoneum in a dose-dependent manner. Both 5αDHB and 5αTHB also suppressed cell infiltration in a dose-dependent manner, and at the highest dose tested, were equally as efficacious as B, indicating that these metabolites retain the anti-inflammatory properties displayed *in vitro* when examined in an *in vivo* model of inflammation. However, it should be noted that full suppression of cell infiltration was not achieved with any of the steroids. Indeed, the highest dose of B achieved a maximal suppression of 45.4 ± 5.0%, indicating that non-glucocorticoid-responsive elements exist within the response to thioglycollate. Previous work with dexamethasone documented <50% suppression of peritoneal cell infiltration with the

single dose used. To our knowledge, there is no work demonstrating that higher doses of dexamethasone achieve greater suppression of cell infiltration.

Given the various cell populations involved in the stages of the inflammatory response, it is important to understand the cell types affected by these steroids. Neutrophils are known to be rapidly recruited to sites of infection, and at the 4h time-point, comprise the largest population of infiltrating cells (Melnicoff *et al.* 1989; Reichardt *et al.* 2001). B suppressed neutrophil infiltration to a similar extent at all doses, suggesting that the maximal suppressive effect of B on neutrophil chemotaxis is achieved at a lower dose than that for suppression of total cells. Both 5αDHB and 5α THB suppressed neutrophil infiltration in a similar manner to B, although here, the most efficacious response was achieved with the highest dose, potentially indicating that the 5α-reduced metabolites are less potent than B. In contrast to the effects on neutrophil infiltration, inflammatory monocyte infiltration, which is elevated 4h-post thioglycollate challenge, was much more responsive to increasing doses of B, which suppressed this cell population in a dose-dependent manner. At the highest dose, B suppressed inflammatory monocytes by 96.4 ± 0.9%, indicating significant effects on this population of cells. Whilst both 5α-reduced metabolites also suppressed the infiltration of inflammatory monocytes, they were significantly less efficacious than B, with 5αDHB achieving 57.3 ± 13.9% suppression and 5αTHB achieving 69.5 ± 12.8% suppression

According to the classical model of acute inflammation, neutrophils are the first population of leukocytes recruited to the site of infection, and release of inflammatory mediators from these infiltrated cells is necessary to initiate the recruitment of monocytes (Melnicoff *et al.* 1989). However, it was recently demonstrated that the recruitment of inflammatory monocytes is an early event in the inflammatory response, and occurs independent of neutrophil migration. Lymphocyte function-associated antigen-1 (LFA-1)-deficient mice, which have significantly inhibited peritoneal neutrophil infiltration, had unaltered recruitment of

inflammatory monocytes compared to wild type mice (Henderson *et al.* 2003). Data in this thesis supports the presence of these inflammatory monocytes early in the inflammatory response, and highlights the ability of both the parent glucocorticoid B and the 5α-reduced glucocorticoid metabolites to suppress the recruitment of this population of leukocytes.

The recruitment of cells into the peritoneum is driven by the release of inflammatory mediators, specifically chemokines, which attract cells to the site of inflammation (Zlotnik and Yoshie 2000). The crucial role of chemokines in models of inflammation is well established. In mouse, neutrophils are recruited in response to the chemokines macrophage inflammatory protein-2 (MIP-2) and keratinocyte chemoattractant (KC) (Zhang *et al.* 2001), whilst monocytes are recruited in response to MCP-1 (Takahashi *et al.* 2009). Previous reports have indicated the critical importance of MCP-1 in monocyte recruitment. Indeed, in MCP-1-deficient mice, numbers of recruited monocytes were significantly reduced compared to wild-type mice following induction of peritonitis (Lu *et al.* 1998). Initially it was believed that recruited neutrophils released MCP-1, driving the recruitment of monocytes. However, the discovery that inflammatory monocyte infiltration occurs independent of neutrophil migration indicated that another cell type was perhaps responsible for MCP-1 release. Indeed, it was demonstrated that resident cells within the peritoneum, specifically resident macrophages and to a lesser extent mesothelial cells, produce the MCP-1 crucial to the recruitment of inflammatory monocytes (Henderson *et al.* 2003). Given the anti-inflammatory effects of 5α-reduced glucocorticoid metabolites on macrophages *in vitro*, it raised the possibility that the anti-inflammatory effects seen *in vivo* were a result of actions upon resident macrophages to suppress MCP-1 release. Analysis of peritoneal lavage revealed that treatment with B significantly reduced MCP-1 levels in a dose-dependent manner, with a similar pattern of suppression to that seen with inflammatory monocyte infiltration, supporting the positive correlation between MCP-1 levels and monocyte recruitment. 5αDHB also suppressed MCP-1 levels, although it was significantly less efficacious than B, again showing a similar pattern to that seen with inflammatory

monocyte recruitment. In contrast to both B and 5αDHB, MCP-1 levels following 5αTHB treatment did not match the pattern of inflammatory monocyte infiltration. Interestingly, 5αTHB had no significant effect on MCP-1 levels, despite reducing inflammatory monocyte recruitment, potentially indicating that 5αTHB affects an alternative chemokine involved in the recruitment of such cells.

MCP-1 also affects neutrophil recruitment. In MCP-1-deficient models of inflammation, neutrophil infiltration into the peritoneum was increased in MCP-1-deficient mice compared to wild-type mice (Takahashi *et al.* 2009). This may potentially explain the lack of dose-dependent changes in neutrophil numbers in our study, despite overall changes in total cell number. It should be noted that the inflammatory monocyte cell population at this time-point is relatively small, comprising less than 5% of all cells. As such, the changes in the number of inflammatory monocytes are likely to contribute only a small amount to the overall changes in the total number of cells. Whilst neutrophils are the predominant population, the lack of dose-dependent change seen in this population with steroid treatment suggests that other cell populations are also affected by glucocorticoids and their 5α-reduced metabolites. Previous work has demonstrated that resident lymphocytes, both T- and B-cells, alter the recruitment of leukocytes to the peritoneum following thioglycollate-injection (Kipari *et al.* 2009). Furthermore, recruitment of lymphocytes into the peritoneum has also been reported (Pitzalis *et al.* 1997). It may be that glucocorticoids alter inflammatory mechanisms within these or other cells to prevent recruitment of lymphocytes, as well as that of leukocytes. Indeed, it has been reported that glucocorticoids inhibit the ability of endothelial cells to bind lymphocytes, thus preventing migration of these cells (Pitzalis *et al.* 1997). To investigate this further, mice deficient in T-cells (NUDE mice) or B-cell (μMT KO mice) could be utilised to establish if these cells play a role in the anti-inflammatory effects of 5α-reduced glucocorticoid metabolites in thioglycollate-induced peritonitis.

Both resident and newly recruited cells have been implicated in the release of pro-inflammatory cytokines following thioglycollate challenge (Henderson *et al.* 2003; Cailhier *et al.* 2005). Having shown that 5α-reduced glucocorticoid metabolites suppress pro-inflammatory cytokine release from macrophages *in vitro*, the ability to do the same *in vivo* was expected. Treatment with B reduced peritoneal levels of IL-6 in a dose-dependent manner. Similar to the suppression of MCP-1, 5αDHB suppressed IL-6 only at the highest dose. 5αTHB also suppressed IL-6, yet this was achieved with only the lowest dose tested. Indeed it appears that increasing the dose of 5αTHB may actually be increasing the levels of IL-6 within the peritoneum, although the effect is not significant. Whilst this is unexpected and is at odds with the reduction in infiltration of inflammatory cells following 5αTHB administration, it does provide support for a potential cell-specific, pro-inflammatory role for 5αTHB that was demonstrated in Chapter 5.

Having seen the anti-inflammatory properties of 5α-reduced glucocorticoid metabolites in suppressing inflammatory signalling in macrophages *in vitro*, and the suppressive effects on the recruitment of inflammatory monocytes *in vivo*, it is clear these metabolites affect cells of the monocyte/macrophage lineage. However, these inflammatory cells are only beginning to appear at the 4h time-point analysed here. It would be of real interest to investigate the effects of these steroids in the later stages of the inflammatory response, namely the resolution phase. In this phase of inflammation, neutrophils undergo apoptosis, preventing excessive local damage from their presence (Heasman *et al.* 2003). Phagocytosis of these apoptotic cells by macrophages ensures their clearance from the inflamed site and prevents the release of their intracellular toxic contents, enabling resolution of the inflammatory response. This process is crucial, with mutant mice showing defects in the clearance of apoptotic cells displaying persistent tissue inflammation (Duffield 2003). Previous work looking at the effects of glucocorticoids on macrophage function demonstrated that glucocorticoids enhance macrophage phagocytosis of apoptotic cells (Liu *et al.* 1999). Further work showed that glucocorticoid treatment of peripheral blood monocytes induced a highly phagocytic monocyte-derived macrophage phenotype

(Giles *et al.* 2001). Such a cell-type may accelerate the resolution phase of the inflammatory response, and demonstrates further anti-inflammatory capacity of glucocorticoids.

Investigation into the effects that 5α-reduced glucocorticoid metabolites have on macrophage phagocytosis, as well as the resolution of inflammation would allow for a much more detailed view of the anti-inflammatory properties of these metabolites. This could be achieved using both an *in vitro* and an *in vivo* approach. Firstly, the ability of 5αDHB and 5αTHB to induce a phagocytic phenotype could be assessed *in vitro* through a phagocytosis assay. Secondly, the effects that 5αDHB and 5αTHB have on the resolution of inflammation could be assessed *in vivo* using the thioglycollate-induced peritonitis model established here. However, in this instance, it would be necessary to analyse the peritoneal exudates at several time points over the course of the peritonitis. Whilst inflammatory monocytes are recruited rapidly within 4 hours of thioglycollate challenge, this is by no means the peak influx of monocytes and macrophages. Indeed, macrophage infiltration rises relatively slowly within the first 12 to 24 hours post-thioglycollate challenge (Henderson *et al.* 2003). Instead, infiltration increases dramatically between 24 and 72 hours post-thioglycollate challenge. It is during this period when macrophages are believed to develop a phagocytic phenotype, removing apoptotic neutrophils from the site of inflammation, thus aiding resolution. As such, it would be extremely interesting to repeat the current experiment, but analyse peritoneal cells at time points beyond the 4 hours tested, specifically 16, 48 and 72 hours post-thioglycollate challenge. In particular, measurement of the levels of apoptosis in the peritoneum throughout such a time course would offer more mechanistic insights into the action of these steroids.

In conclusion, these results demonstrate for the first time the anti-inflammatory properties of 5α-reduced glucocorticoid metabolites *in vivo*. Treatment of mice with 5αDHB and 5αTHB resulted in inhibition of thioglycollate-induced cell infiltration into the peritoneum, a similar result to that achieved with the parent glucocorticoid

B. The effects of these steroids on cell infiltration suggest that they may be acting through resident peritoneal cells to inhibit recruitment of neutrophils and inflammatory monocytes. Further use of this model will allow for a clearer understanding of the anti-inflammatory mechanism of these 5α-reduced glucocorticoid metabolites. Furthermore, both 5α-reduced glucocorticoid metabolites suppressed the levels of IL-6 in the peritoneum of thioglycollate-treated mice, although to a lesser extent than B. However, these findings provide exciting evidence that the anti-inflammatory effects of 5α-reduced glucocorticoid metabolites demonstrated *in vitro* are retained *in vivo*, and so these metabolites may potentially be 'safer' alternatives to standard glucocorticoid therapy in the treatment of inflammatory conditions.

Chapter 7

Summary and Future Work

The central theme of this thesis is that glucocorticoid metabolism alters the inflammatory state evident in obesity, and vice versa. The aims were to investigate the effects of anti-inflammatory salicylate treatment on glucocorticoid metabolism in obesity and the anti-inflammatory properties of 5α-reduced glucocorticoid metabolites. The work described in this thesis demonstrates that the insulin sensitising effects of salicylate are dependent upon 11βHSD1 and suggests that this enzyme plays a role in regulating the inflammatory state of obese adipose tissue. Furthermore, this thesis demonstrates that both *in vitro* and *in vivo*, 5α-reduced glucocorticoid metabolites have similar anti-inflammatory properties to the parent glucocorticoid, indicating that increased hepatic A-ring metabolism in obesity is not a pathway of simple inactivation.

Obesity rates are increasing in almost all countries, with 1.5 billion adults worldwide predicted to be overweight or obese by 2015 by the WHO. With this rise comes increased incidence of insulin resistance, type 2 diabetes and cardiovascular disease, factors which together have been termed the 'metabolic syndrome.' Given the increased mortality rates amongst obese individuals, as well as the economic burden due to healthcare, much emphasis has been placed on achieving a greater understanding of the mechanisms underlying the development and progression of the metabolic syndrome, as well as novel therapeutic strategies.

Under normal conditions, feeding results in the release of insulin from pancreatic β-cells, which acts to promote the uptake and non-oxidative metabolism of glucose in adipose, liver and muscle, as well as inhibiting hepatic gluconeogenesis and glycogenolysis (White 2002). Glucocorticoids act in catabolic manner, such that in times of starvation, the body is primed to respond. In doing so, glucocorticoids act to counter-regulate the actions of insulin, whose actions are inhibited through disruption of insulin signalling pathways (Macfarlane *et al.* 2008). The role that glucocorticoids play in energy homeostasis is most dramatically exemplified in conditions of glucocorticoid deficiency or excess, namely Addison's disease and Cushing's

syndrome respectively (Boscaro *et al.* 2001; Kyriazopoulou 2007). Patients with Cushing's syndrome display strikingly similar symptoms to patients with metabolic syndrome, with visceral obesity, hypertension, dyslipidemia and insulin resistance. Those with Cushing's have pronounced hypersecretion of glucocorticoids primarily due to the presence of pituitary adenomas, thus raising circulating levels (Boscaro *et al.* 2001). In contrast, metabolic syndrome does not present with elevated circulating levels of glucocorticoids (Walker 2007). However, there are distinct, tissue-specific changes in pre-receptor metabolism of glucocorticoids in obesity and the associated metabolic syndrome. Adipose levels of the glucocorticoid-regenerating enzyme 11βHSD1 are elevated in obesity (Wake *et al.* 2003; Livingstone *et al.* 2005), elevating local levels of glucocorticoids, whilst hepatic levels of 5αR1, an enzyme which reduces glucocorticoids to their 5α-metabolites, are increased (Livingstone *et al.* 2005). Whilst these alterations have been characterised, much remains to be elucidated about both the regulation of these enzymes, as well as the consequences of their altered expression.

The role that inflammation plays in obesity-related disorders has been an area of intensive research since the discovery that the expression of pro-inflammatory cytokines was increased in obese adipose tissue (Hotamisligil *et al.* 1995). In terms of adipose tissue, the general hypothesis involves the development of adipocyte hypertrophy in response to chronic over-nutrition. This provokes the infiltration of macrophages, which subsequently become activated and release pro-inflammatory cytokines, thus initiating a cycle of inflammation. Recently it was demonstrated, *in vitro*, that pro-inflammatory cytokines induce expression of 11βHSD1 in both adipocytes and macrophages (Ishii *et al.* 2007; Ishii-Yonemoto *et al.* 2010), potentially explaining the elevated levels of this enzyme in obese adipose tissue. In this thesis, we have demonstrated an *in vivo* link between adipose inflammation and glucocorticoid metabolism in obesity. Treatment of DIO mice with the anti-inflammatory drug sodium salicylate improved insulin sensitivity and reduced markers of adipose inflammation in visceral adipose. These changes were accompanied by a downregulation of 11βHSD1 in the same visceral adipose depots,

demonstrating an association between inflammation and 11βHSD1 specifically within visceral adipose. The utilisation of 11βHSD1-deficient mice allowed us to determine if 11βHSD1 downregulation was crucial to the insulin sensitising effects of salicylate. In these 11βHSD1-deficient mice, salicylate treatment did not recapitulate the insulin sensitising effects observed in wild type mice, revealing that the downregulation of visceral adipose 11βHSD1 is a central factor in the ability of anti-inflammatory agents to improve insulin sensitivity.

Whilst this work has clearly demonstrated a link between obese adipose inflammation and glucocorticoid metabolism dysregulation, several intriguing aspects remain to be fully addressed. Firstly, one must consider the tissue-specific nature of the effects, given that the majority of alterations, in terms of inflammatory mediators and 11βHSD1, as well as lipid metabolism, occurred in visceral adipose. Accumulating evidence indicates that visceral adipose is more metabolically active than peripheral adipose depots, and clear differences exist between adipose depots in terms of both inflammatory state and glucocorticoid action (Wajchenberg 2000). The ability of salicylates to reduce expression of 11βHSD1 is likely a consequence of their suppressive actions of the inflammation within these adipose depots, and so by reducing the levels of pro-inflammatory cytokines, the induction of 11βHSD1 is reduced. However, this leads onto a further question that remains to be elucidated, that of 11βHSD1 regulation. Given that pro-inflammatory cytokines upregulate 11βHSD1, the above hypothesis that salicylates reduce 11βHSD1 indirectly through suppression of inflammation is an attractive one, yet one that has yet to be fully investigated. Indeed, it is possible that salicylates act directly to alter transcriptional regulation of 11βHSD1 and this is an area that needs to be addressed. To date, the only reported direct regulators of 11βHSD1 gene transcription are members of the CCAAT/enhancer-binding protein (C/EBP) transcription factor family (Sai *et al.* 2008). Recent unpublished data in a human fibroblast cell line has shown that pro-inflammatory cytokines upregulate mRNA levels of C/EBPβ in accordance with increases in 11βHSD1 mRNA levels, whilst knockdown of C/EBPβ inhibited the increase in 11βHSD1 expression (Róg-Zielinska 2009). Therefore, it would be

interesting to assess the impact that salicylate has directly on the mRNA expression of not only 11βHSD1, but also transcription factors of the C/EBP family. Furthermore, the use of chromatin immunoprecipitation (ChIP) analysis, which enables assessment of the interactions between proteins and DNA, would provide crucial information as to whether C/EBPβ binds to the promoter region of 11βHSD1 following salicylate treatment, as well as other transcription factors potentially involved in the regulation of 11βHSD1. Such data could provide new mechanistic insights into links between inflammation and glucocorticoid metabolism.

A further issue involves the apparent paradoxical situation within obese adipose tissue, whereby there is an increase in the levels of an enzyme that generates steroids known for their anti-inflammatory properties, yet there is also an increase in inflammation. Whilst this appears counter-intuitive, one must consider that the well characterised anti-inflammatory properties of glucocorticoids are seen primarily at pharmacological levels. Much less is known about the effects of physiological levels of endogenous glucocorticoids, although there are indications of a pro-inflammatory role for glucocorticoids. A recent study showed TNFα induced an increase in 11βHSD1 in pre-adipocytes, augmenting the release of pro-inflammatory mediators (Ishii-Yonemoto *et al.* 2010). Furthermore, treatment with 11βHSD1 inhibitors reduced TNFα-induced release of pro-inflammatory mediators. Such data presents an intriguing situation whereby increasing local glucocorticoid levels through increased 11βHSD1 actually intensifies inflammation. An especially interesting point from this study was that overexpression of 11βHSD1 using a targeted vector did not enhance inflammation (Ishii-Yonemoto *et al.* 2010). This suggests that the ability of 11βHSD1-mediated glucocorticoid to induce inflammation is dependent upon a pre-existing inflammatory state. Furthermore, one must take into account the differences in the chronic, low-grade, inflammatory state seen in obesity with that seen in acute inflammatory conditions, such as thioglycollate-induced peritonitis. In acute inflammation, an immune response is mounted against a foreign body, whether it be pathogen or allergen, until it is eliminated, at which point the immune response is downgraded. However, in chronic inflammation, whilst the body mounts an initial

response to an insult, the continuous stimulation of inflammatory pathways prevents the resolution of the response, resulting in persistent inflammation.

The accumulation of this evidence clearly establishes that the pathways involved in the development of insulin resistance from inflammation and glucocorticoid excess are highly complex and by no means linear in nature. It may be the case that an initial inflammatory insult in adipose, possibly provided by hypertrophied adipocytes, begins a cycle in which 11βHSD1 expression is increased in adipocytes and stromovascular cells, including macrophages and pre-adipocytes. This local amplification of glucocorticoids serves to enhance the local inflammatory response, as well as inducing metabolic effects, both of which serve to induce an insulin resistant state. Certainly, future investigations are necessary to unravel the intracellular mechanisms at work within obese adipose tissue.

The demonstration that hepatic levels of 5αR1 are elevated in obesity ignited interest in the consequences of changes in this route of glucocorticoid metabolism (Livingstone *et al.* 2000). Initially viewed as a pathway of inactivation, this hypothesis was challenged when it was revealed that 5α-reduced metabolites of the rodent glucocorticoid corticosterone, namely 5αDHB and 5αTHB, are capable of binding the glucocorticoid receptor (GR) (McInnes *et al.* 2004). Further work has since shown that 5αTHB does not potently induce transactivation of glucocorticoid-responsive metabolic genes such as PEPCK and TAT, but is capable of suppressing pro-inflammatory cytokine release, demonstrating a transrepressive ability (Yang 2009). This evidence suggested that 5αTHB was a potential 'dissociated' steroid that retained the anti-inflammatory effects of the parent steroid, but lacks to the metabolic effects. The development of 'dissociated' steroids has become a principal aim within glucocorticoid research, given that the pharmacological doses required to control inflammation in patients are often coupled with the development of detrimental side effects (Schacke *et al.* 2002).

In this thesis, the aim was to investigate the molecular mechanisms behind the potential anti-inflammatory properties of both 5αDHB and 5αTHB, and how they compared to those of the parent glucocorticoid. Whilst both 5αDHB and 5αTHB suppress pro-inflammatory cytokine release in macrophages, the mechanisms underlying this suppression, whilst similar, display distinct differences. In particular, the ability to induce transactivation of anti-inflammatory proteins was different between the two metabolites. 5αDHB induced expression of MKP-1 and IκBα, whilst 5αTHB only increased expression of MKP-1. This is intriguing as the mechanisms of transactivation differ between MKP-1 and IκBα differ, with MKP-1 shown to be induced by GR monomer binding (Abraham *et al.* 2006), whilst IκBα requires GR dimerisation (Heck *et al.* 1997). This suggests that 5αTHB is unable to induce GR dimerisation, and so is unable to induce transactivation of genes that require GR dimerisation, including IκBα, PEPCK and TAT. This is an area that certainly warrants further investigation and one that would yield invaluable knowledge into the actions of these 5α-reduced glucocorticoid metabolites. One approach could be to utilise *in vitro* techniques with reporter plasmids that are activated through either GR monomers or GR dimers. Specifically, one could use the glucocorticoid-responsive PNMT reporter, which has been shown to be activated in GR dimerisation-deficient mice (Adams *et al.* 2003), and a more conventional glucocorticoid-responsive reporter that requires dimerisation, such as the MMTV reporter (Michailidou *et al.* 2008), and compare the effects of each 5α-reduced glucocorticoid metabolite.

Whilst both 5α-reduced glucocorticoid metabolites exerted similar effects in macrophages, the same was not observed in a different cell line. In kidney cells, 5αDHB suppressed activation of pro-inflammatory transcription factors in a similar manner to corticosterone, again demonstrating potent anti-inflammatory effect. However, 5αTHB augmented pro-inflammatory transcription factor activation. This presents the intriguing issue of cell-specific effects of these metabolites, in particular 5αTHB. More research is certainly required here to elucidate the mechanisms behind this.

Given the anti-inflammatory effects of these 5α-reduced metabolites witnessed in macrophages *in vitro*, it was important to establish if a similar story unfolded when they were tested in an *in vivo* model of inflammation. Here we demonstrated that both 5αDHB and 5αTHB suppress the influx of cells to the site of inflammation, in particular neutrophils and inflammatory monocytes, as well as reducing the local levels of IL-6. This data reveals for the first time *in vivo* that 5α-reduced glucocorticoid metabolites retain the anti-inflammatory properties of the parent glucocorticoid, and appear similarly efficacious in terms of suppression of total cell infiltration. This is particularly pertinent when one considers the cell-specific effects seen *in vitro* of 5αTHB. Given those results, it may have been expected that 5αTHB may not exhibit such an anti-inflammatory effect in an *in vivo* setting. Furthermore, the indication that 5αTHB is unable to induce GR dimerisation may also have been expected to dampen potential suppressive effects on inflammation. Yet in terms of ability to suppress cell infiltration, 5αTHB was similarly efficacious to corticosterone. This is perhaps an indication that induction of anti-inflammatory molecules through GR dimerisation-dependent mechanisms has little overall anti-inflammatory effect. Indeed, previous work has been shown that glucocorticoids are capable of suppressing an inflammatory response in GR dimerisation-deficient mice just as effectively as in wild type mice (Reichardt *et al.* 1998; Reichardt *et al.* 2001).

However, there were still distinct differences evident between the parent glucocorticoid and the metabolites. This was particularly apparent for the infiltration of inflammatory monocytes, as well as the release of IL-6 and MCP-1, in which in all cases, corticosterone was markedly more efficacious than both 5αDHB and 5αTHB. The effects on inflammatory monocytes are intriguing and are an area that would benefit from further investigation as these cells are only beginning to enter the inflammatory site at the time point tested (Henderson *et al.* 2003). Analysis at later time points may reveal further effects on the differentiation of these monocytes into macrophages, which would have implications for the resolution of the inflammatory response, as well as highlighting differences between the parent glucocorticoid and the 5α-reduced metabolites. However, this data provides an initial first-step into the

potential application of 5α-reduced glucocorticoid metabolites as an alternative anti-inflammatory treatment to conventional glucocorticoids.

In terms of the role these metabolites play in obesity, very little is currently known. The increase in hepatic 5αR1 seen in obesity was initially viewed as increased clearance of local glucocorticoids, thus protecting the liver from their adverse metabolic effects. Whilst this appears to be accurate, it fails to deal with the prospect of increased levels of 5α-reduced glucocorticoid metabolites. In terms of immunomodulatory effects, data in this thesis indicates that 5αDHB retains the anti-inflammatory properties of corticosterone, and given that this was evident in two distinct cell types, as well as *in vivo,* it lends to the theory that 5αDHB would also act in an anti-inflammatory manner in the liver. The cell-specific effects of 5αTHB demonstrated *in vitro* make it difficult to establish the potential effects of this metabolite on inflammation within the liver. Yet the *in vivo* data suggests that within a whole body, 5αTHB exerts anti-inflammatory effects. Investigation utilising a liver cell line would provide important insights into the hepatic effects of these metabolites. Use of such an *in* vitro system would also allow for a greater understanding of the possible metabolic effects, or lack thereof, of these metabolites. Certainly in terms of 5αTHB, previous work has indicated a reduced effect on metabolic gene induction compared to the parent glucocorticoid (Yang 2009). No data currently exists on the metabolic effects of 5αDHB, and this is clearly an issue that needs to be addressed. A further issue is that of metabolism of these steroids. For example, whether the results from treatment with 5αDHB are truly 5αDHB-dependent or whether they are a result of its metabolism to 5αTHB and subsequent action. We addressed this issue *in vitro* by characterising the cell types used for the presence of A-ring enzymes, demonstrating that HEK293 cells lack A-ring metabolising enzymes, whilst RAW264.7 cells only express 5αR1. Therefore, it appears that the effects are specific for the steroid administered. This could be further addressed by measuring the levels of these metabolites within cells, potentially via gas chromatography-mass spectroscopy, thus ensuring conversion to another form is not responsible for effects.

Nonetheless, data presented in this thesis indicates that aside from simply 'clearing' local glucocorticoids, 5α-reduction actively generates glucocorticoid metabolites that exert distinct and cell-specific effects, perhaps revealing an endogenous pathway of dissociation. Whilst this thesis has dealt with possible consequences of A-ring metabolism, the issue of 5αR1 regulation remains an area yet to be fully explored. The presence of hepatic inflammation in obesity is likely to play a role in 5αR1 expression, yet this has yet to be tested. However, given the ability of pro-inflammatory cytokines to induce 11βHSD1 expression, it suggests that increased pro-inflammatory mediators in the liver may act to induce 5αR1.

Figure 7.1 provides a simplified overview of possible pathways at work within the body in response to obesity, although several of the interactions remain to be investigated. Data in this thesis suggests that the inflammation associated with obesity has a key role to play in the regulation of glucocorticoid metabolism. The upregulation in 11βHSD1 will enhance local levels of glucocorticoid which will act upon metabolic pathways, and in the case of adipose tissue, may also act to enhance inflammation, resulting in the development of insulin resistance. Whilst inflammatory mediators have not been directly shown to regulate 5αR1, the elevated presence of both these in obesity indicates a possible link exists. By metabolising local glucocorticoids, hepatic 5αR1 increases the levels of 5α-reduced glucocorticoid metabolites, which have the anti-inflammatory properties of the parent glucocorticoid, but not the adverse metabolic effects, potentially resulting in a dampening of hepatic inflammation and suppression of the development of insulin resistance.

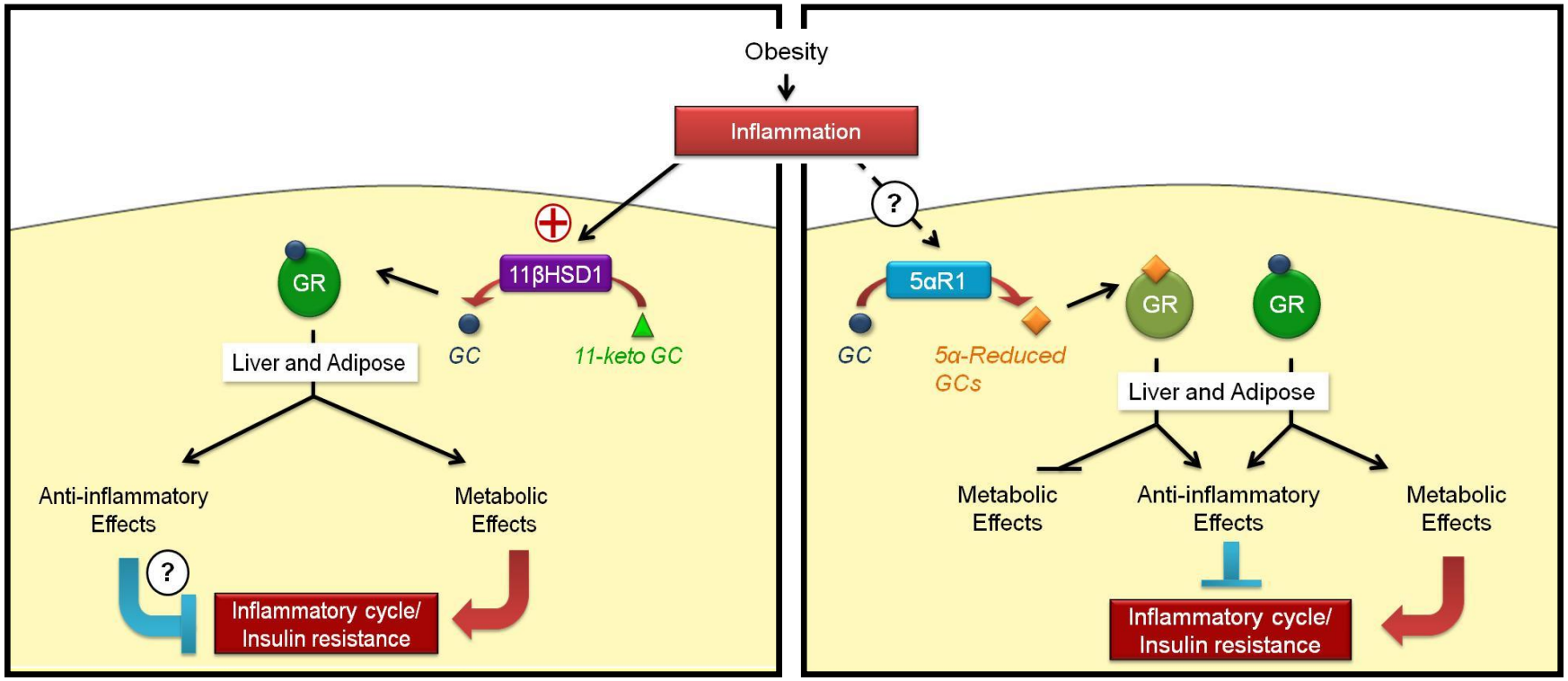

Figure 7.1 Proposed interactions between inflammation and glucocorticoid metabolism in obesity. Left panel; inflammation seen in the obese state results in the upregulation of adipose 11βHSD1, increasing local glucocorticoid (GC) levels, which act through the GC receptor (GR) to activate metabolic pathways, resulting in the development of insulin resistance. Whilst classically seen as anti-inflammatory, it is debatable whether the regenerated GCs in adipose are acting to dampen or exacerbate the inflammatory response. Right panel; obesity-induced inflammation may potentially upregulate hepatic levels of 5αR1, generating greater levels of 5α-reduced GC metabolites. Whilst these 5α-reduced metabolites retain anti-inflammatory properties, they lack the adverse metabolic effects of the parent GC. Therefore, upregulation of 5αR1 may be an adaptive response to limit hepatic exposure to GCs.

References

Abbasi, F., J. W. Chu, C. Lamendola, T. McLaughlin, J. Hayden, G. M. Reaven and P. D. Reaven (2004). Discrimination between obesity and insulin resistance in the relationship with adiponectin. *Diabetes* **53**(3): 585-90.

Abraham, S. M., T. Lawrence, A. Kleiman, P. Warden, M. Medghalchi, J. Tuckermann, J. Saklatvala and A. R. Clark (2006). Antiinflammatory effects of dexamethasone are partly dependent on induction of dual specificity phosphatase 1. *J Exp Med* **203**(8): 1883-9.

Adams, M., O. C. Meijer, J. Wang, A. Bhargava and D. Pearce (2003). Homodimerization of the glucocorticoid receptor is not essential for response element binding: activation of the phenylethanolamine N-methyltransferase gene by dimerization-defective mutants. *Mol Endocrinol* **17**(12): 2583-92.

Aguirre, V., T. Uchida, L. Yenush, R. Davis and M. F. White (2000). The c-Jun NH(2)-terminal kinase promotes insulin resistance during association with insulin receptor substrate-1 and phosphorylation of Ser(307). *J Biol Chem* **275**(12): 9047-54.

Ajuwon, K. M. and M. E. Spurlock (2005). Adiponectin inhibits LPS-induced NF-kappaB activation and IL-6 production and increases PPARgamma2 expression in adipocytes. *Am J Physiol Regul Integr Comp Physiol* **288**(5): R1220-5.

Al-Dujaili, E. A., B. C. Williams and C. R. Edwards (1981). The development and application of a direct radioimmunoassay for corticosterone. *Steroids* **37**(2): 157-76.

Alangari, A. A. (2010). Genomic and non-genomic actions of glucocorticoids in asthma. *Ann Thorac Med* **5**(3): 133-9.

Alberti, K. G., P. Zimmet and J. Shaw (2005). The metabolic syndrome--a new worldwide definition. *Lancet* **366**(9491): 1059-62.

Alberts, P., L. Engblom, N. Edling, M. Forsgren, G. Klingstrom, C. Larsson, Y. Ronquist-Nii, B. Ohman and L. Abrahmsen (2002). Selective inhibition of 11beta-hydroxysteroid dehydrogenase type 1 decreases blood glucose concentrations in hyperglycaemic mice. *Diabetologia* **45**(11): 1528-32.

Alberts, P., C. Nilsson, G. Selen, L. O. Engblom, N. H. Edling, S. Norling, G. Klingstrom, C. Larsson, M. Forsgren, M. Ashkzari, C. E. Nilsson, M. Fiedler, E. Bergqvist, B. Ohman, E. Bjorkstrand and L. B. Abrahmsen (2003). Selective inhibition of 11 beta-hydroxysteroid dehydrogenase type 1 improves hepatic insulin sensitivity in hyperglycemic mice strains. *Endocrinology* **144**(11): 4755-62.

Andersson, S., D. M. Berman, E. P. Jenkins and D. W. Russell (1991). Deletion of steroid 5 alpha-reductase 2 gene in male pseudohermaphroditism. *Nature* **354**(6349): 159-61.

Andersson, S. and D. W. Russell (1990). Structural and biochemical properties of cloned and expressed human and rat steroid 5 alpha-reductases. *Proc Natl Acad Sci U S A* **87**(10): 3640-4.

Andrew, R., D. I. Phillips and B. R. Walker (1998). Obesity and gender influence cortisol secretion and metabolism in man. *J Clin Endocrinol Metab* **83**(5): 1806-9.

Andrews, R. C. and B. R. Walker (1999). Glucocorticoids and insulin resistance: old hormones, new targets. *Clin Sci (Lond)* **96**(5): 513-23.

Arita, Y., S. Kihara, N. Ouchi, M. Takahashi, K. Maeda, J. Miyagawa, K. Hotta, I. Shimomura, T. Nakamura, K. Miyaoka, H. Kuriyama, M. Nishida, S. Yamashita, K. Okubo, K. Matsubara, M. Muraguchi, Y. Ohmoto, T. Funahashi and Y. Matsuzawa (1999). Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity. *Biochem Biophys Res Commun* **257**(1): 79-83.

Arkan, M. C., A. L. Hevener, F. R. Greten, S. Maeda, Z. W. Li, J. M. Long, A. Wynshaw-Boris, G. Poli, J. Olefsky and M. Karin (2005). IKK-beta links inflammation to obesity-induced insulin resistance. *Nat Med* **11**(2): 191-8.

Askew, E. B., R. T. Gampe, Jr., T. B. Stanley, J. L. Faggart and E. M. Wilson (2007). Modulation of androgen receptor activation function 2 by testosterone and dihydrotestosterone. *J Biol Chem* **282**(35): 25801-16.

Barnes, P. J. (1998). Anti-inflammatory actions of glucocorticoids: molecular mechanisms. *Clin Sci (Lond)* **94**(6): 557-72.

Barnes, P. J. (2010). Mechanisms and resistance in glucocorticoid control of inflammation. *J Steroid Biochem Mol Biol* **120**(2-3): 76-85.

Bartholome, B., C. M. Spies, T. Gaber, S. Schuchmann, T. Berki, D. Kunkel, M. Bienert, A. Radbruch, G. R. Burmester, R. Lauster, A. Scheffold and F. Buttgereit (2004). Membrane glucocorticoid receptors (mGCR) are expressed in normal human peripheral blood mononuclear cells and up-regulated after in vitro stimulation and in patients with rheumatoid arthritis. *FASEB J* **18**(1): 70-80.

Barzilai, N., L. She, B. Q. Liu, P. Vuguin, P. Cohen, J. Wang and L. Rossetti (1999). Surgical removal of visceral fat reverses hepatic insulin resistance. *Diabetes* **48**(1): 94-8.

Bastard, J. P., M. Maachi, J. T. Van Nhieu, C. Jardel, E. Bruckert, A. Grimaldi, J. J. Robert, J. Capeau and B. Hainque (2002). Adipose tissue IL-6 content correlates with resistance to insulin activation of glucose uptake both in vivo and in vitro. *J Clin Endocrinol Metab* **87**(5): 2084-9.

Beck, I. M., W. Vanden Berghe, L. Vermeulen, K. R. Yamamoto, G. Haegeman and K. De Bosscher (2009). Crosstalk in inflammation: the interplay of glucocorticoid receptor-based mechanisms and kinases and phosphatases. *Endocr Rev* **30**(7): 830-82.

Beyaert, R., G. Van Loo, K. Heyninck and P. Vandenabeele (2002). Signaling to gene activation and cell death by tumor necrosis factor receptors and Fas. *Int Rev Cytol* **214**: 225-72.

Bezaire, V. and D. Langin (2009). Regulation of adipose tissue lipolysis revisited. *Proc Nutr Soc*: 1-11.

Bizzi, A. and L. A. Carlson (1965). Stimulatory effect of ethanol on glycerol release from rat adipose tissue in vitro. *Life Sci* **4**(21): 2123-8.

Bjorntorp, P. (1992). Abdominal fat distribution and the metabolic syndrome. *J Cardiovasc Pharmacol* **20 Suppl 8**: S26-8.

Bjorntorp, P. (1995). Endocrine abnormalities of obesity. *Metabolism* **44**(9 Suppl 3): 21-3.

Bladh, L. G., J. Liden, K. Dahlman-Wright, M. Reimers, S. Nilsson and S. Okret (2005). Identification of endogenous glucocorticoid repressed genes differentially regulated by a glucocorticoid receptor mutant able to separate between nuclear factor-kappaB and activator protein-1 repression. *Mol Pharmacol* **67**(3): 815-26.

Bledsoe, R. K., V. G. Montana, T. B. Stanley, C. J. Delves, C. J. Apolito, D. D. McKee, T. G. Consler, D. J. Parks, E. L. Stewart, T. M. Willson, M. H. Lambert, J. T. Moore, K. H. Pearce and H. E. Xu (2002). Crystal structure of the glucocorticoid receptor ligand binding domain reveals a novel mode of receptor dimerization and coactivator recognition. *Cell* **110**(1): 93-105.

Blumberg, B., W. Sabbagh, Jr., H. Juguilon, J. Bolado, Jr., C. M. van Meter, E. S. Ong and R. M. Evans (1998). SXR, a novel steroid and xenobiotic-sensing nuclear receptor. *Genes Dev* **12**(20): 3195-205.

Boscaro, M., L. Barzon, F. Fallo and N. Sonino (2001). Cushing's syndrome. *Lancet* **357**(9258): 783-91.

Bost, F., M. Aouadi, L. Caron and B. Binetruy (2005). The role of MAPKs in adipocyte differentiation and obesity. *Biochimie* **87**(1): 51-6.

Bratusch-Marrain, P. R., H. Vierhapper, M. Komjati and W. K. Waldhausl (1985). Acetyl-salicylic acid impairs insulin-mediated glucose utilization and reduces insulin clearance in healthy and non-insulin-dependent diabetic man. *Diabetologia* **28**(9): 671-6.

Brem, A. S. (2001). Insights into glucocorticoid-associated hypertension. *Am J Kidney Dis* **37**(1): 1-10.

Brichard, S. M., M. L. Delporte and M. Lambert (2003). Adipocytokines in anorexia nervosa: a review focusing on leptin and adiponectin. *Horm Metab Res* **35**(6): 337-42.

Buren, J., H. X. Liu, J. Jensen and J. W. Eriksson (2002). Dexamethasone impairs insulin signalling and glucose transport by depletion of insulin receptor substrate-1, phosphatidylinositol 3-kinase and protein kinase B in primary cultured rat adipocytes. *Eur J Endocrinol* **146**(3): 419-29.

Cai, D., M. Yuan, D. F. Frantz, P. A. Melendez, L. Hansen, J. Lee and S. E. Shoelson (2005). Local and systemic insulin resistance resulting from hepatic activation of IKK-beta and NF-kappaB. *Nat Med* **11**(2): 183-90.

Cailhier, J. F., M. Partolina, S. Vuthoori, S. Wu, K. Ko, S. Watson, J. Savill, J. Hughes and R. A. Lang (2005). Conditional macrophage ablation demonstrates that resident macrophages initiate acute peritoneal inflammation. *J Immunol* **174**(4): 2336-42.

Calandra, T., J. Bernhagen, C. N. Metz, L. A. Spiegel, M. Bacher, T. Donnelly, A. Cerami and R. Bucala (1995). MIF as a glucocorticoid-induced modulator of cytokine production. *Nature* **377**(6544): 68-71.

Camps, M., A. Nichols and S. Arkinstall (2000). Dual specificity phosphatases: a gene family for control of MAP kinase function. *FASEB J* **14**(1): 6-16.

Cancello, R., C. Henegar, N. Viguerie, S. Taleb, C. Poitou, C. Rouault, M. Coupaye, V. Pelloux, D. Hugol, J. L. Bouillot, A. Bouloumie, G. Barbatelli, S. Cinti, P. A. Svensson, G. S. Barsh, J. D. Zucker, A. Basdevant, D. Langin and K. Clement (2005). Reduction of macrophage infiltration and chemoattractant gene expression changes in white adipose tissue of morbidly obese subjects after surgery-induced weight loss. *Diabetes* **54**(8): 2277-86.

Cancello, R., J. Tordjman, C. Poitou, G. Guilhem, J. L. Bouillot, D. Hugol, C. Coussieu, A. Basdevant, A. Bar Hen, P. Bedossa, M. Guerre-Millo and K. Clement (2006). Increased infiltration of macrophages in omental adipose tissue is associated with marked hepatic lesions in morbid human obesity. *Diabetes* **55**(6): 1554-61.

Carlstedt-Duke, J., J. A. Gustafsson, S. A. Gustafsson and O. Wrange (1977). Interactions of corticosterone, 5alpha-dihydrocorticosterone and

dexamethasone with proteins in rat-liver cytosol. *Eur J Biochem* **73**(1): 231-8.

Carter, R. N., J. M. Paterson, U. Tworowska, D. J. Stenvers, J. J. Mullins, J. R. Seckl and M. C. Holmes (2009). Hypothalamic-pituitary-adrenal axis abnormalities in response to deletion of 11beta-HSD1 is strain-dependent. *J Neuroendocrinol* **21**(11): 879-87.

Cash, J., G. White and D. Greaves (2009). Chapter 17. Zymosan-induced peritonitis as a simple experimental system for the study of inflammation. *Methods in enzymology* **461**: 379-396.

Chapman, K. E., A. E. Coutinho, M. Gray, J. S. Gilmour, J. S. Savill and J. R. Seckl (2009). The role and regulation of 11beta-hydroxysteroid dehydrogenase type 1 in the inflammatory response. *Mol Cell Endocrinol* **301**(1-2): 123-31.

Chen, D., A. Carpenter, J. Abrahams, R. C. Chambers, R. I. Lechler, J. H. McVey and A. Dorling (2008). Protease-activated receptor 1 activation is necessary for monocyte chemoattractant protein 1-dependent leukocyte recruitment in vivo. *J Exp Med* **205**(8): 1739-46.

Chen, H. (2006). Cellular inflammatory responses: novel insights for obesity and insulin resistance. *Pharmacol Res* **53**(6): 469-77.

Cho, I. J. and S. G. Kim (2009). A novel mitogen-activated protein kinase phosphatase-1 and glucocorticoid receptor (GR) interacting protein-1-dependent combinatorial mechanism of gene transrepression by GR. *Mol Endocrinol* **23**(1): 86-99.

Chung, B. C., J. Picado-Leonard, M. Haniu, M. Bienkowski, P. F. Hall, J. E. Shively and W. L. Miller (1987). Cytochrome P450c17 (steroid 17 alpha-hydroxylase/17,20 lyase): cloning of human adrenal and testis cDNAs indicates the same gene is expressed in both tissues. *Proc Natl Acad Sci U S A* **84**(2): 407-11.

Cinti, S., G. Mitchell, G. Barbatelli, I. Murano, E. Ceresi, E. Faloia, S. Wang, M. Fortier, A. S. Greenberg and M. S. Obin (2005). Adipocyte death defines macrophage localization and function in adipose tissue of obese mice and humans. *J Lipid Res* **46**(11): 2347-55.

Clark, A. R. (2007). Anti-inflammatory functions of glucocorticoid-induced genes. *Mol Cell Endocrinol* **275**(1-2): 79-97.

Coghlan, M. J., P. B. Jacobson, B. Lane, M. Nakane, C. W. Lin, S. W. Elmore, P. R. Kym, J. R. Luly, G. W. Carter, R. Turner, C. M. Tyree, J. Hu, M. Elgort, J. Rosen and J. N. Miner (2003). A novel antiinflammatory maintains glucocorticoid efficacy with reduced side effects. *Mol Endocrinol* **17**(5): 860-9.

Cooper, M. S. and P. M. Stewart (2009). 11β-Hydroxysteroid dehydrogenase type 1 and its role in the hypothalamus-pituitary-adrenal axis, metabolic syndrome, and inflammation. *J Clin Endocrinol Metab*.

Coppack, S. W., M. D. Jensen and J. M. Miles (1994). In vivo regulation of lipolysis in humans. *J Lipid Res* **35**(2): 177-93.

Croxtall, J. D., Q. Choudhury and R. J. Flower (2000). Glucocorticoids act within minutes to inhibit recruitment of signalling factors to activated EGF receptors through a receptor-dependent, transcription-independent mechanism. *Br J Pharmacol* **130**(2): 289-98.

Dandona, P., R. Weinstock, K. Thusu, E. Abdel-Rahman, A. Aljada and T. Wadden (1998). Tumor necrosis factor-alpha in sera of obese patients: fall with weight loss. *J Clin Endocrinol Metab* **83**(8): 2907-10.

De Bosscher, K. (2010). Selective Glucocorticoid Receptor modulators. *J Steroid Biochem Mol Biol* **120**(2-3): 96-104.

De Bosscher, K. and G. Haegeman (2009). Minireview: latest perspectives on antiinflammatory actions of glucocorticoids. *Mol Endocrinol* **23**(3): 281-91.

De Bosscher, K., M. L. Schmitz, W. Vanden Berghe, S. Plaisance, W. Fiers and G. Haegeman (1997). Glucocorticoid-mediated repression of nuclear factor-kappaB-dependent transcription involves direct interference with transactivation. *Proc Natl Acad Sci U S A* **94**(25): 13504-9.

De Bosscher, K., W. Vanden Berghe, I. M. Beck, W. Van Molle, N. Hennuyer, J. Hapgood, C. Libert, B. Staels, A. Louw and G. Haegeman (2005). A fully dissociated compound of plant origin for inflammatory gene repression. *Proc Natl Acad Sci U S A* **102**(44): 15827-32.

De Bosscher, K., W. Vanden Berghe and G. Haegeman (2003). The interplay between the glucocorticoid receptor and nuclear factor-kappaB or activator protein-1: molecular mechanisms for gene repression. *Endocr Rev* **24**(4): 488-522.

De Matteo, R. and C. N. May (1997). Glucocorticoid-induced renal vasodilatation is mediated by a direct renal action involving nitric oxide. *Am J Physiol* **273**(6 Pt 2): R1972-9.

Dewint, P., V. Gossye, K. De Bosscher, W. Vanden Berghe, K. Van Beneden, D. Deforce, S. Van Calenbergh, U. Muller-Ladner, B. Vander Cruyssen, G. Verbruggen, G. Haegeman and D. Elewaut (2008). A plant-derived ligand favoring monomeric glucocorticoid receptor conformation with impaired transactivation potential attenuates collagen-induced arthritis. *J Immunol* **180**(4): 2608-15.

Doering, D. D., S. Steckelbroeck, T. Doering and D. Klingmuller (2002). Effects of butyltins on human 5alpha-reductase type 1 and type 2 activity. *Steroids* **67**(10): 859-67.

Dostert, A. and T. Heinzel (2004). Negative glucocorticoid receptor response elements and their role in glucocorticoid action. *Curr Pharm Des* **10**(23): 2807-16.

Draznin, B. (2006). Molecular mechanisms of insulin resistance: serine phosphorylation of insulin receptor substrate-1 and increased expression of p85alpha: the two sides of a coin. *Diabetes* **55**(8): 2392-7.

Duffield, J. S. (2003). The inflammatory macrophage: a story of Jekyll and Hyde. *Clin Sci (Lond)* **104**(1): 27-38.

Ehrchen, J., L. Steinmuller, K. Barczyk, K. Tenbrock, W. Nacken, M. Eisenacher, U. Nordhues, C. Sorg, C. Sunderkotter and J. Roth (2007). Glucocorticoids induce differentiation of a specifically activated, anti-inflammatory subtype of human monocytes. *Blood* **109**(3): 1265-74.

Fain, J. N. (2010). Release of inflammatory mediators by human adipose tissue is enhanced in obesity and primarily by the nonfat cells: a review. *Mediators Inflamm* **2010**: 513948.

Falardeau, P. and A. Martineau (1989). Prostaglandin I2 and glucocorticoid-induced rise in arterial pressure in the rat. *J Hypertens* **7**(8): 625-32.

Fallo, F., A. Scarda, N. Sonino, A. Paoletta, M. Boscaro, C. Pagano, G. Federspil and R. Vettor (2004). Effect of glucocorticoids on adiponectin: a study in healthy subjects and in Cushing's syndrome. *Eur J Endocrinol* **150**(3): 339-44.

Fant, M. E., J. Yeakley and R. W. Harrison (1983). Evidence for carrier-mediated transport of glucocorticoids by human placental membrane vesicles. *Biochim Biophys Acta* **731**(3): 415-20.

Fasshauer, M., J. Klein, S. Neumann, M. Eszlinger and R. Paschke (2002). Hormonal regulation of adiponectin gene expression in 3T3-L1 adipocytes. *Biochem Biophys Res Commun* **290**(3): 1084-9.

Fernandez-Real, J.-M., A. Lopez-Bermejo, A.-B. Ropero, S. Piquer, A. Nadal, J. Bassols, R. Casamitjana, R. Gomis, E. Arnaiz, I. Perez and W. Ricart (2008). Salicylates increase insulin secretion in healthy obese subjects. *J Clin Endocrinol Metab* **93**(7): 2523-2530.

Fleischman, A., S. E. Shoelson, R. Bernier and A. B. Goldfine (2008). Salsalate improves glycemia and inflammatory parameters in obese young adults. *Diabetes Care* **31**(2): 289-94.

Fontana, L., J. C. Eagon, M. E. Trujillo, P. E. Scherer and S. Klein (2007). Visceral fat adipokine secretion is associated with systemic inflammation in obese humans. *Diabetes* **56**(4): 1010-3.

Frayn, K. N., F. Karpe, B. A. Fielding, I. A. Macdonald and S. W. Coppack (2003). Integrative physiology of human adipose tissue. *Int J Obes Relat Metab Disord* **27**(8): 875-88.

Freedman, N. D. and K. R. Yamamoto (2004). Importin 7 and importin alpha/importin beta are nuclear import receptors for the glucocorticoid receptor. *Mol Biol Cell* **15**(5): 2276-86.

Gao, Z., D. Hwang, F. Bataille, M. Lefevre, D. York, M. J. Quon and J. Ye (2002). Serine phosphorylation of insulin receptor substrate 1 by inhibitor kappa B kinase complex. *J Biol Chem* **277**(50): 48115-21.

Garg, A. (2004). Regional adiposity and insulin resistance. *J Clin Endocrinol Metab* **89**(9): 4206-10.

George, F. W. (1997). Androgen metabolism in the prostate of the finasteride-treated, adult rat: a possible explanation for the differential action of testosterone and 5 alpha-dihydrotestosterone during development of the male urogenital tract. *Endocrinology* **138**(3): 871-7.

Ghali, S. A., B. Gottlieb, R. Lumbroso, L. K. Beitel, Y. Elhaji, J. Wu, L. Pinsky and M. A. Trifiro (2003). The use of androgen receptor amino/carboxyl-terminal interaction assays to investigate androgen receptor gene mutations in subjects with varying degrees of androgen insensitivity. *J Clin Endocrinol Metab* **88**(5): 2185-93.

Gilbert, K. C. and N. J. Brown (2010). Aldosterone and inflammation. *Curr Opin Endocrinol Diabetes Obes* **17**(3): 199-204.

Giles, K. M., K. Ross, A. G. Rossi, N. A. Hotchin, C. Haslett and I. Dransfield (2001). Glucocorticoid augmentation of macrophage capacity for phagocytosis of apoptotic cells is associated with reduced p130Cas expression, loss of paxillin/pyk2 phosphorylation, and high levels of active Rac. *J Immunol* **167**(2): 976-86.

Goebeler, M., R. Gillitzer, K. Kilian, K. Utzel, E. B. Brocker, U. R. Rapp and S. Ludwig (2001). Multiple signaling pathways regulate NF-kappaB-dependent transcription of the monocyte chemoattractant protein-1 gene in primary endothelial cells. *Blood* **97**(1): 46-55.

Goldfine, A. B., V. Fonseca, K. A. Jablonski, L. Pyle, M. A. Staten and S. E. Shoelson (2010). The effects of salsalate on glycemic control in patients with type 2 diabetes: a randomized trial. *Ann Intern Med* **152**(6): 346-57.

Goldfine, A. B., R. Silver, W. Aldhahi, D. Cai, E. Tatro, J. Lee and S. E. Shoelson (2008). Use of salsalate to target inflammation in the treatment of insulin resistance and type 2 diabetes. *Clin Transl Sci* **1**(1): 36-43.

Griffin, M. E., M. J. Marcucci, G. W. Cline, K. Bell, N. Barucci, D. Lee, L. J. Goodyear, E. W. Kraegen, M. F. White and G. I. Shulman (1999). Free fatty acid-induced insulin resistance is associated with activation of protein kinase C theta and alterations in the insulin signaling cascade. *Diabetes* **48**(6): 1270-4.

Harman-Boehm, I., M. Bluher, H. Redel, N. Sion-Vardy, S. Ovadia, E. Avinoach, I. Shai, N. Kloting, M. Stumvoll, N. Bashan and A. Rudich (2007). Macrophage infiltration into omental versus subcutaneous fat across different populations: effect of regional adiposity and the comorbidities of obesity. *J Clin Endocrinol Metab* **92**(6): 2240-7.

Hayden, M. S. and S. Ghosh (2008). Shared principles in NF-kappaB signaling. *Cell* **132**(3): 344-62.

Hazzalin, C. A. and L. C. Mahadevan (2002). MAPK-regulated transcription: a continuously variable gene switch? *Nat Rev Mol Cell Biol* **3**(1): 30-40.

He, B., R. T. Gampe, Jr., A. T. Hnat, J. L. Faggart, J. T. Minges, F. S. French and E. M. Wilson (2006). Probing the functional link between androgen receptor coactivator and ligand-binding sites in prostate cancer and androgen insensitivity. *J Biol Chem* **281**(10): 6648-63.

Heasman, S. J., K. M. Giles, C. Ward, A. G. Rossi, C. Haslett and I. Dransfield (2003). Glucocorticoid-mediated regulation of granulocyte apoptosis and macrophage phagocytosis of apoptotic cells: implications for the resolution of inflammation. *J Endocrinol* **178**(1): 29-36.

Heck, S., K. Bender, M. Kullmann, M. Gottlicher, P. Herrlich and A. C. Cato (1997). I kappaB alpha-independent downregulation of NF-kappaB activity by glucocorticoid receptor. *Embo J* **16**(15): 4698-707.

Hector, J., B. Schwarzloh, J. Goehring, T. G. Strate, U. F. Hess, G. Deuretzbacher, N. Hansen-Algenstaedt, F. U. Beil and P. Algenstaedt (2007). TNF-alpha alters visfatin and adiponectin levels in human fat. *Horm Metab Res* **39**(4): 250-5.

Hench, P. S., E. C. Kendall and et al. (1949). The effect of a hormone of the adrenal cortex (17-hydroxy-11-dehydrocorticosterone; compound E) and of pituitary adrenocorticotropic hormone on rheumatoid arthritis. *Mayo Clin Proc* **24**(8): 181-97.

Henderson, R. B., J. A. Hobbs, M. Mathies and N. Hogg (2003). Rapid recruitment of inflammatory monocytes is independent of neutrophil migration. *Blood* **102**(1): 328-35.

Hirosumi, J., G. Tuncman, L. Chang, C. Z. Gorgun, K. T. Uysal, K. Maeda, M. Karin and G. S. Hotamisligil (2002). A central role for JNK in obesity and insulin resistance. *Nature* **420**(6913): 333-6.

Hotamisligil, G. S. (2006). Inflammation and metabolic disorders. *Nature* **444**(7121): 860-7.

Hotamisligil, G. S., P. Arner, J. F. Caro, R. L. Atkinson and B. M. Spiegelman (1995). Increased adipose tissue expression of tumor necrosis factor-alpha in human obesity and insulin resistance. *J Clin Invest* **95**(5): 2409-15.

Hotamisligil, G. S., N. S. Shargill and B. M. Spiegelman (1993). Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. *Science* **259**(5091): 87-91.

Hundal, R. S., K. F. Petersen, A. B. Mayerson, P. S. Randhawa, S. Inzucchi, S. E. Shoelson and G. I. Shulman (2002). Mechanism by which high-dose aspirin improves glucose metabolism in type 2 diabetes. *J Clin Invest* **109**(10): 1321-6.

Inouye, K. E., H. Shi, J. K. Howard, C. H. Daly, G. M. Lord, B. J. Rollins and J. S. Flier (2007). Absence of CC Chemokine Ligand 2 does not limit obesity-associated infiltration of macrophages into adipose tissue. *Diabetes* **56**(9): 2242-2250.

Ishii-Yonemoto, T., H. Masuzaki, S. Yasue, S. Okada, C. Kozuka, T. Tanaka, M. Noguchi, T. Tomita, J. Fujikura, Y. Yamamoto, K. Ebihara, K. Hosoda and K. Nakao (2010). Glucocorticoid reamplification within cells intensifies NF-κB and MAPK signaling and reinforces inflammation in activated preadipocytes. *Am J Physiol Endocrinol Metab* **298**(5): E930-940.

Ishii, T., H. Masuzaki, T. Tanaka, N. Arai, S. Yasue, N. Kobayashi, T. Tomita, M. Noguchi, J. Fujikura, K. Ebihara, K. Hosoda and K. Nakao (2007). Augmentation of 11beta-hydroxysteroid dehydrogenase type 1 in LPS-activated J774.1 macrophages--role of 11beta-HSD1 in pro-inflammatory properties in macrophages. *FEBS Lett* **581**(3): 349-54.

Ito, A., T. Suganami, Y. Miyamoto, Y. Yoshimasa, M. Takeya, Y. Kamei and Y. Ogawa (2007). Role of MAPK phosphatase-1 in the induction of monocyte chemoattractant protein-1 during the course of adipocyte hypertrophy. *J Biol Chem* **282**(35): 25445-52.

Jackman, R. W. and S. C. Kandarian (2004). The molecular basis of skeletal muscle atrophy. *Am J Physiol Cell Physiol* **287**(4): C834-43.

Jiang, W. and D. S. Pisetsky (2008). The induction of HMGB1 release from RAW 264.7 cells by transfected DNA. *Mol Immunol* **45**(7): 2038-44.

Johnson, G. L. and R. Lapadat (2002). Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. *Science* **298**(5600): 1911-2.

Kadowaki, T., T. Yamauchi, N. Kubota, K. Hara, K. Ueki and K. Tobe (2006). Adiponectin and adiponectin receptors in insulin resistance, diabetes, and the metabolic syndrome. *J Clin Invest* **116**(7): 1784-92.

Kamada, Y., T. Takehara and N. Hayashi (2008). Adipocytokines and liver disease. *J Gastroenterol* **43**(11): 811-22.

Kamei, N., K. Tobe, R. Suzuki, M. Ohsugi, T. Watanabe, N. Kubota, N. Ohtsuka-Kowatari, K. Kumagai, K. Sakamoto, M. Kobayashi, T. Yamauchi, K. Ueki, Y. Oishi, S. Nishimura, I. Manabe, H. Hashimoto, Y. Ohnishi, H. Ogata, K. Tokuyama, M. Tsunoda, T. Ide, K. Murakami, R. Nagai and T. Kadowaki (2006). Overexpression of monocyte chemoattractant protein-1 in adipose tissues causes macrophage recruitment and insulin resistance. *J Biol Chem* **281**(36): 26602-14.

Kanda, H., S. Tateya, Y. Tamori, K. Kotani, K. Hiasa, R. Kitazawa, S. Kitazawa, H. Miyachi, S. Maeda, K. Egashira and M. Kasuga (2006). MCP-1 contributes to macrophage infiltration into adipose tissue, insulin resistance, and hepatic steatosis in obesity. *J Clin Invest* **116**(6): 1494-505.

Karpe, F. and G. D. Tan (2005). Adipose tissue function in the insulin-resistance syndrome. *Biochem Soc Trans* **33**(Pt 5): 1045-8.

Kassel, O., A. Sancono, J. Kratzschmar, B. Kreft, M. Stassen and A. C. Cato (2001). Glucocorticoids inhibit MAP kinase via increased expression and decreased degradation of MKP-1. *Embo J* **20**(24): 7108-16.

Kenyon, C., Saccoccia, N. (1985). The effects of long-term infusions of the reduced derivatives of aldosterone on water and electrolyte metabolism. *The Adrenal Gland and Hypertension* **27**: 209-214.

Kershaw, E. E. and J. S. Flier (2004). Adipose tissue as an endocrine organ. *J Clin Endocrinol Metab* **89**(6): 2548-56.

Khanna, M., K. N. Qin, R. W. Wang and K. C. Cheng (1995). Substrate specificity, gene structure, and tissue-specific distribution of multiple human 3 alpha-hydroxysteroid dehydrogenases. *J Biol Chem* **270**(34): 20162-8.

Kim, J. K., Y. J. Kim, J. J. Fillmore, Y. Chen, I. Moore, J. Lee, M. Yuan, Z. W. Li, M. Karin, P. Perret, S. E. Shoelson and G. I. Shulman (2001). Prevention of fat-induced insulin resistance by salicylate. *J Clin Invest* **108**(3): 437-46.

Kipari, T., S. Watson, K. Houlberg, S. Lepage, J. Hughes and J. F. Cailhier (2009). Lymphocytes modulate peritoneal leukocyte recruitment in peritonitis. *Inflamm Res* **58**(9): 553-60.

Klein, S., D. B. Allison, S. B. Heymsfield, D. E. Kelley, R. L. Leibel, C. Nonas and R. Kahn (2007). Waist circumference and cardiometabolic risk: a consensus statement from Shaping America's Health: Association for Weight Management and Obesity Prevention; NAASO, The Obesity Society; the American Society for Nutrition; and the American Diabetes Association. *Am J Clin Nutr* **85**(5): 1197-202.

Klover, P. J., A. H. Clementi and R. A. Mooney (2005). Interleukin-6 depletion selectively improves hepatic insulin action in obesity. *Endocrinology* **146**(8): 3417-27.

Kopp, E. and S. Ghosh (1994). Inhibition of NF-kappa B by sodium salicylate and aspirin. *Science* **265**(5174): 956-9.

Kopp, H. P., C. W. Kopp, A. Festa, K. Krzyzanowska, S. Kriwanek, E. Minar, R. Roka and G. Schernthaner (2003). Impact of weight loss on inflammatory proteins and their association with the insulin resistance syndrome in morbidly obese patients. *Arterioscler Thromb Vasc Biol* **23**(6): 1042-7.

Koska, J., E. Ortega, J. C. Bunt, A. Gasser, J. Impson, R. L. Hanson, J. Forbes, B. de Courten and J. Krakoff (2009). The effect of salsalate on insulin action and glucose tolerance in obese non-diabetic patients: results of a randomised double-blind placebo-controlled study. *Diabetologia* **52**(3): 385-93.

Kotelevtsev, Y., M. C. Holmes, A. Burchell, P. M. Houston, D. Schmoll, P. Jamieson, R. Best, R. Brown, C. R. Edwards, J. R. Seckl and J. J. Mullins (1997). 11beta-Hydroxysteroid dehydrogenase type 1 knockout mice show attenuated glucocorticoid-inducible responses and resist hyperglycemia on obesity or stress. *Proc Natl Acad Sci U S A* **94**(26): 14924-9.

Kumar, R. and E. B. Thompson (2005). Gene regulation by the glucocorticoid receptor: structure:function relationship. *J Steroid Biochem Mol Biol* **94**(5): 383-94.

Kyriazopoulou, V. (2007). Glucocorticoid replacement therapy in patients with Addison's disease. *Expert Opin Pharmacother* **8**(6): 725-9.

Lavery, G. G., E. A. Walker, N. Draper, P. Jeyasuria, J. Marcos, C. H. Shackleton, K. L. Parker, P. C. White and P. M. Stewart (2006). Hexose-6-phosphate dehydrogenase knock-out mice lack 11 beta-hydroxysteroid dehydrogenase type 1-mediated glucocorticoid generation. *J Biol Chem* **281**(10): 6546-51.

Lee, C. G., M. C. Carr, S. J. Murdoch, E. Mitchell, N. F. Woods, M. H. Wener, W. L. Chandler, E. J. Boyko and J. D. Brunzell (2009). Adipokines, inflammation, and visceral adiposity across the menopausal transition: a prospective study. *J Clin Endocrinol Metab* **94**(4): 1104-10.

Leng, L., W. Wang, T. Roger, M. Merk, M. Wuttke, T. Calandra and R. Bucala (2009). Glucocorticoid-induced MIF expression by human CEM T cells. *Cytokine* **48**(3): 177-85.

Lin, H. K., C. F. Hung, M. Moore and T. M. Penning (1999). Genomic structure of rat 3alpha-hydroxysteroid/dihydrodiol dehydrogenase (3alpha-HSD/DD, AKR1C9). *J Steroid Biochem Mol Biol* **71**(1-2): 29-39.

Lindsay, R. S., D. J. Wake, S. Nair, J. Bunt, D. E. W. Livingstone, P. A. Permana, P. A. Tataranni and B. R. Walker (2003). Subcutaneous adipose 11β-hydroxysteroid dehydrogenase type 1 activity and messenger ribonucleic acid levels are associated with adiposity and insulinemia in Pima Indians and Caucasians. *J Clin Endocrinol Metab* **88**(6): 2738-2744.

Liu, Y., J. M. Cousin, J. Hughes, J. Van Damme, J. R. Seckl, C. Haslett, I. Dransfield, J. Savill and A. G. Rossi (1999). Glucocorticoids promote nonphlogistic phagocytosis of apoptotic leukocytes. *J Immunol* **162**(6): 3639-46.

Livingstone, D. (2008). Increased susceptibility to liver fat accumulation and insulin resistance, but not obesity, induced by high fat diet in 5 alpha-reductase type 1 knock-out mice. *Proceeding of American Endocrine Society* P2-41.

Livingstone, D. E., G. C. Jones, K. Smith, P. M. Jamieson, R. Andrew, C. J. Kenyon and B. R. Walker (2000). Understanding the role of glucocorticoids in obesity: tissue-specific alterations of corticosterone metabolism in obese Zucker rats. *Endocrinology* **141**(2): 560-3.

Livingstone, D. E., K. J. McInnes, B. R. Walker and R. Andrew (2005). Increased A-ring reduction of glucocorticoids in obese Zucker rats: effects of insulin sensitization. *Obes Res* **13**(9): 1523-6.

Loll, P. J., D. Picot and R. M. Garavito (1995). The structural basis of aspirin activity inferred from the crystal structure of inactivated prostaglandin H2 synthase. *Nat Struct Biol* **2**(8): 637-43.

Lu, B., B. J. Rutledge, L. Gu, J. Fiorillo, N. W. Lukacs, S. L. Kunkel, R. North, C. Gerard and B. J. Rollins (1998). Abnormalities in monocyte recruitment and cytokine expression in monocyte chemoattractant protein 1-deficient mice. *J Exp Med* **187**(4): 601-8.

Lu, N. Z., S. E. Wardell, K. L. Burnstein, D. Defranco, P. J. Fuller, V. Giguere, R. B. Hochberg, L. McKay, J. M. Renoir, N. L. Weigel, E. M. Wilson, D. P. McDonnell and J. A. Cidlowski (2006). International Union of

Pharmacology. LXV. The pharmacology and classification of the nuclear receptor superfamily: glucocorticoid, mineralocorticoid, progesterone, and androgen receptors. *Pharmacol Rev* **58**(4): 782-97.

Lupien, S. J., M. de Leon, S. de Santi, A. Convit, C. Tarshish, N. P. Nair, M. Thakur, B. S. McEwen, R. L. Hauger and M. J. Meaney (1998). Cortisol levels during human aging predict hippocampal atrophy and memory deficits. *Nat Neurosci* **1**(1): 69-73.

Luu-The, V., G. Pelletier and F. Labrie (2005). Quantitative appreciation of steroidogenic gene expression in mouse tissues: new roles for type 2 5alpha-reductase, 20alpha-hydroxysteroid dehydrogenase and estrogen sulfotransferase. *J Steroid Biochem Mol Biol* **93**(2-5): 269-76.

Macfarlane, D. P., S. Forbes and B. R. Walker (2008). Glucocorticoids and fatty acid metabolism in humans: fuelling fat redistribution in the metabolic syndrome. *J Endocrinol* **197**(2): 189-204.

MacPhee, I. A., F. A. Antoni and D. W. Mason (1989). Spontaneous recovery of rats from experimental allergic encephalomyelitis is dependent on regulation of the immune system by endogenous adrenal corticosteroids. *J Exp Med* **169**(2): 431-45.

Maeda, N., I. Shimomura, K. Kishida, H. Nishizawa, M. Matsuda, H. Nagaretani, N. Furuyama, H. Kondo, M. Takahashi, Y. Arita, R. Komuro, N. Ouchi, S. Kihara, Y. Tochino, K. Okutomi, M. Horie, S. Takeda, T. Aoyama, T. Funahashi and Y. Matsuzawa (2002). Diet-induced insulin resistance in mice lacking adiponectin/ACRP30. *Nat Med* **8**(7): 731-7.

Majewska, M. D. (1987). Antagonist-type interaction of glucocorticoids with the GABA receptor-coupled chloride channel. *Brain Res* **418**(2): 377-82.

Marin, P., B. Andersson, M. Ottosson, L. Olbe, B. Chowdhury, H. Kvist, G. Holm, L. Sjostrom and P. Bjorntorp (1992). The morphology and metabolism of intraabdominal adipose tissue in men. *Metabolism* **41**(11): 1242-8.

Martinez, F. O., S. Gordon, M. Locati and A. Mantovani (2006). Transcriptional profiling of the human monocyte-to-macrophage differentiation and polarization: new molecules and patterns of gene expression. *J Immunol* **177**(10): 7303-11.

Masuzaki, H., J. Paterson, H. Shinyama, N. M. Morton, J. J. Mullins, J. R. Seckl and J. S. Flier (2001). A transgenic model of visceral obesity and the metabolic syndrome. *Science* **294**(5549): 2166-70.

Mathieu, P., I. Lemieux and J. P. Despres (2010). Obesity, inflammation, and cardiovascular risk. *Clin Pharmacol Ther* **87**(4): 407-16.

Matthews, L., A. Berry, V. Ohanian, J. Ohanian, H. Garside and D. Ray (2008). Caveolin mediates rapid glucocorticoid effects and couples glucocorticoid action to the antiproliferative program. *Mol Endocrinol* **22**(6): 1320-30.

Maury, E. and S. M. Brichard (2010). Adipokine dysregulation, adipose tissue inflammation and metabolic syndrome. *Mol Cell Endocrinol* **314**(1): 1-16.

McEwen, B. S., C. A. Biron, K. W. Brunson, K. Bulloch, W. H. Chambers, F. S. Dhabhar, R. H. Goldfarb, R. P. Kitson, A. H. Miller, R. L. Spencer and J. M. Weiss (1997). The role of adrenocorticoids as modulators of immune function in health and disease: neural, endocrine and immune interactions. *Brain Res Brain Res Rev* **23**(1-2): 79-133.

McInnes, K. J., C. J. Kenyon, K. E. Chapman, D. E. Livingstone, L. J. Macdonald, B. R. Walker and R. Andrew (2004). 5alpha-Reduced glucocorticoids, novel endogenous activators of the glucocorticoid receptor. *J Biol Chem* **279**(22): 22908-12.

McMaster, A. and D. W. Ray (2007). Modelling the glucocorticoid receptor and producing therapeutic agents with anti-inflammatory effects but reduced side-effects. *Exp Physiol* **92**(2): 299-309.

Medzhitov, R. (2008). Origin and physiological roles of inflammation. *Nature* **454**(7203): 428-35.

Melnicoff, M. J., P. K. Horan and P. S. Morahan (1989). Kinetics of changes in peritoneal cell populations following acute inflammation. *Cell Immunol* **118**(1): 178-91.

Meyer, T., J. A. Gustafsson and J. Carlstedt-Duke (1997). Glucocorticoid-dependent transcriptional repression of the osteocalcin gene by competitive binding at the TATA box. *DNA Cell Biol* **16**(8): 919-27.

Michailidou, Z., R. N. Carter, E. Marshall, H. G. Sutherland, D. G. Brownstein, E. Owen, K. Cockett, V. Kelly, L. Ramage, E. A. Al-Dujaili, M. Ross, I. Maraki, K. Newton, M. C. Holmes, J. R. Seckl, N. M. Morton, C. J. Kenyon and K. E. Chapman (2008). Glucocorticoid receptor haploinsufficiency causes hypertension and attenuates hypothalamic-pituitary-adrenal axis and blood pressure adaptions to high-fat diet. *FASEB J* **22**(11): 3896-907.

Mitchell, B. M. and R. C. Webb (2002). Impaired vasodilation and nitric oxide synthase activity in glucocorticoid-induced hypertension. *Biol Res Nurs* **4**(1): 16-21.

Mitic, T. (2009). The role of murine 11beta HSD1 in the metabolism of 7-oxysterols, University of Edinburgh.

Mohlig, M., M. Freudenberg, T. Bobbert, M. Ristow, H. Rochlitz, M. O. Weickert, A. F. Pfeiffer and J. Spranger (2006). Acetylsalicylic acid improves lipid-induced insulin resistance in healthy men. *J Clin Endocrinol Metab* **91**(3): 964-7.

Montesinos, M. C., A. Desai and B. N. Cronstein (2006). Suppression of inflammation by low-dose methotrexate is mediated by adenosine A2A receptor but not A3 receptor activation in thioglycollate-induced peritonitis. *Arthritis Res Ther* **8**(2): R53.

Monzillo, L. U., O. Hamdy, E. S. Horton, S. Ledbury, C. Mullooly, C. Jarema, S. Porter, K. Ovalle, A. Moussa and C. S. Mantzoros (2003). Effect of lifestyle modification on adipokine levels in obese subjects with insulin resistance. *Obes Res* **11**(9): 1048-54.

Morton, N. M. (2009). Obesity and corticosteroids: 11beta-hydroxysteroid type 1 as a cause and therapeutic target in metabolic disease. *Mol Cell Endocrinol* **316**(2): 154-64.

Morton, N. M., M. C. Holmes, C. Fievet, B. Staels, A. Tailleux, J. J. Mullins and J. R. Seckl (2001). Improved lipid and lipoprotein profile, hepatic insulin sensitivity, and glucose tolerance in 11beta-hydroxysteroid dehydrogenase type 1 null mice. *J Biol Chem* **276**(44): 41293-300.

Morton, N. M., J. M. Paterson, H. Masuzaki, M. C. Holmes, B. Staels, C. Fievet, B. R. Walker, J. S. Flier, J. J. Mullins and J. R. Seckl (2004). Novel adipose tissue-mediated resistance to diet-induced visceral obesity in 11beta-hydroxysteroid dehydrogenase type 1-deficient mice. *Diabetes* **53**(4): 931-8.

Morton, N. M. and J. R. Seckl (2008). 11beta-Hydroxysteroid dehydrogenase type 1 and obesity. *Front Horm Res* **36**: 146-64.

Muller, E. and H. H. Kerschbaum (2006). Progesterone and its metabolites 5-dihydroprogesterone and 5-3-tetrahydroprogesterone decrease LPS-induced NO release in the murine microglial cell line, BV-2. *Neuro Endocrinol Lett* **27**(5): 675-8.

Murano, I., G. Barbatelli, V. Parisani, C. Latini, G. Muzzonigro, M. Castellucci and S. Cinti (2008). Dead adipocytes, detected as crown-like structures, are prevalent in visceral fat depots of genetically obese mice. *J Lipid Res* **49**(7): 1562-8.

Nathan, C. (2002). Points of control in inflammation. *Nature* **420**(6917): 846-52.

Netea, M. G., C. J. Tack, P. M. Netten, J. A. Lutterman and J. W. Van der Meer (2001). The effect of salicylates on insulin sensitivity. *J Clin Invest* **108**(11): 1723-4.

Newman, W. P. and R. G. Brodows (1983). Aspirin causes tissue insensitivity to insulin in normal man. *J Clin Endocrinol Metab* **57**(6): 1102-6.

Newton, R. and N. S. Holden (2007). Separating transrepression and transactivation: a distressing divorce for the glucocorticoid receptor? *Mol Pharmacol* **72**(4): 799-809.

Nguyen-Duy, T. B., M. Z. Nichaman, T. S. Church, S. N. Blair and R. Ross (2003). Visceral fat and liver fat are independent predictors of metabolic risk factors in men. *Am J Physiol Endocrinol Metab* **284**(6): E1065-71.

Nishi, M. and M. Kawata (2007). Dynamics of glucocorticoid receptor and mineralocorticoid receptor: implications from live cell imaging studies. *Neuroendocrinology* **85**(3): 186-92.

Nissen, R. M. and K. R. Yamamoto (2000). The glucocorticoid receptor inhibits NFkappaB by interfering with serine-2 phosphorylation of the RNA polymerase II carboxy-terminal domain. *Genes Dev* **14**(18): 2314-29.

Normington, K. and D. W. Russell (1992). Tissue distribution and kinetic characteristics of rat steroid 5 alpha-reductase isozymes. Evidence for distinct physiological functions. *J Biol Chem* **267**(27): 19548-54.

O'Neill, L. A. (2008). The interleukin-1 receptor/Toll-like receptor superfamily: 10 years of progress. *Immunol Rev* **226**: 10-8.

Odermatt, A., P. Arnold and F. J. Frey (2001). The intracellular localization of the mineralocorticoid receptor is regulated by 11beta-hydroxysteroid dehydrogenase type 2. *J Biol Chem* **276**(30): 28484-92.

Okuda, A. and K. Okuda (1984). Purification and characterization of delta 4-3-ketosteroid 5 beta-reductase. *J Biol Chem* **259**(12): 7519-24.

Olefsky, J. M. and C. K. Glass (2010). Macrophages, inflammation, and insulin resistance. *Annu Rev Physiol* **72**: 219-46.

Olson, A. L., J. E. Robillard, C. T. Kisker, B. A. Smith and S. Perlman (1991). Negative regulation of angiotensinogen gene expression by glucocorticoids in fetal sheep liver. *Pediatr Res* **30**(3): 256-60.

Ozcan, U., Q. Cao, E. Yilmaz, A. H. Lee, N. N. Iwakoshi, E. Ozdelen, G. Tuncman, C. Gorgun, L. H. Glimcher and G. S. Hotamisligil (2004). Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes. *Science* **306**(5695): 457-61.

Palermo, M., M. G. Marazzi, B. A. Hughes, P. M. Stewart, P. T. Clayton and C. H. Shackleton (2008). Human Delta4-3-oxosteroid 5beta-reductase (AKR1D1) deficiency and steroid metabolism. *Steroids* **73**(4): 417-23.

Park, E., V. Wong, X. Guan, A. I. Oprescu and A. Giacca (2007). Salicylate prevents hepatic insulin resistance caused by short-term elevation of free fatty acids in vivo. *J Endocrinol* **195**(2): 323-31.

Paterson, J. M., N. M. Morton, C. Fievet, C. J. Kenyon, M. C. Holmes, B. Staels, J. R. Seckl and J. J. Mullins (2004). Metabolic syndrome without obesity: Hepatic overexpression of 11beta-hydroxysteroid dehydrogenase type 1 in transgenic mice. *Proc Natl Acad Sci U S A* **101**(18): 7088-93.

Penning, T. M. (1999). Molecular determinants of steroid recognition and catalysis in aldo-keto reductases. Lessons from 3alpha-hydroxysteroid dehydrogenase. *J Steroid Biochem Mol Biol* **69**(1-6): 211-25.

Pitzalis, C., N. Pipitone, G. Bajocchi, M. Hall, N. Goulding, A. Lee, G. Kingsley, J. Lanchbury and G. Panayi (1997). Corticosteroids inhibit lymphocyte binding to endothelium and intercellular adhesion: an additional mechanism for their anti-inflammatory and immunosuppressive effect. *J Immunol* **158**(10): 5007-16.

Pitzalis, C., N. Pipitone and M. Perretti (2002). Regulation of leukocyte-endothelial interactions by glucocorticoids. *Ann N Y Acad Sci* **966**: 108-18.

Poniachik, J., A. Csendes, J. C. Diaz, J. Rojas, P. Burdiles, F. Maluenda, G. Smok, R. Rodrigo and L. A. Videla (2006). Increased production of IL-1alpha and TNF-alpha in lipopolysaccharide-stimulated blood from obese patients with non-alcoholic fatty liver disease. *Cytokine* **33**(5): 252-7.

Pratt, W. B., Y. Morishima, M. Murphy and M. Harrell (2006). Chaperoning of glucocorticoid receptors. *Handb Exp Pharmacol*(172): 111-38.

Rask, E., T. Olsson, S. Soderberg, R. Andrew, D. E. Livingstone, O. Johnson and B. R. Walker (2001). Tissue-specific dysregulation of cortisol metabolism in human obesity. *J Clin Endocrinol Metab* **86**(3): 1418-21.

Reichardt, H. M., K. H. Kaestner, J. Tuckermann, O. Kretz, O. Wessely, R. Bock, P. Gass, W. Schmid, P. Herrlich, P. Angel and G. Schutz (1998). DNA binding of the glucocorticoid receptor is not essential for survival. *Cell* **93**(4): 531-41.

Reichardt, H. M., J. P. Tuckermann, M. Gottlicher, M. Vujic, F. Weih, P. Angel, P. Herrlich and G. Schutz (2001). Repression of inflammatory responses in the absence of DNA binding by the glucocorticoid receptor. *Embo J* **20**(24): 7168-73.

Reid, J., A. I. Macdougall and M. M. Andrews (1957). Aspirin and diabetes mellitus. *Br Med J* **2**(5053): 1071-4.

Renga, B., M. Migliorati, A. Mencarelli and S. Fiorucci (2009). Reciprocal regulation of the bile acid-activated receptor FXR and the interferon-gamma-STAT-1 pathway in macrophages. *Biochim Biophys Acta* **1792**(6): 564-73.

Ribon, V., J. H. Johnson, H. S. Camp and A. R. Saltiel (1998). Thiazolidinediones and insulin resistance: peroxisome proliferatoractivated receptor gamma activation stimulates expression of the CAP gene. *Proc Natl Acad Sci U S A* **95**(25): 14751-6.

Robertson, S., F. Allie-Reid, W. Vanden Berghe, K. Visser, A. Binder, D. Africander, M. Vismer, K. De Bosscher, J. Hapgood, G. Haegeman and A. Louw (2010). Abrogation of glucocorticoid receptor dimerization correlates with dissociated glucocorticoid behavior of compound a. *J Biol Chem* **285**(11): 8061-75.

Róg-Zielinska, E. (2009). Pro-inflammatory cytokine induction of 11β-hydroxysteroid dehydrogenase-1 requires the action of C/EBPβ, University of Edinburgh. **Thesis for Masters by Research**.

Roland, B. L., Z. S. Krozowski and J. W. Funder (1995). Glucocorticoid receptor, mineralocorticoid receptors, 11 beta-hydroxysteroid dehydrogenase-1 and -2 expression in rat brain and kidney: in situ studies. *Mol Cell Endocrinol* **111**(1): R1-7.

Rosas, M., B. Thomas, M. Stacey, S. Gordon and P. R. Taylor (2010). The myeloid 7/4-antigen defines recently generated inflammatory macrophages and is synonymous with Ly-6B. *J Leukoc Biol* **88**(1): 169-80.

Rosenstock, J., S. Banarer, V. A. Fonseca, S. E. Inzucchi, W. Sun, W. Yao, G. Hollis, R. Flores, R. Levy, W. V. Williams, J. R. Seckl and R. Huber (2010). The 11-beta-hydroxysteroid dehydrogenase type 1 inhibitor INCB13739 improves hyperglycemia in patients with type 2 diabetes inadequately controlled by metformin monotherapy. *Diabetes Care* **33**(7): 1516-22.

Roszak, A. W., Y. A. Lefebvre, G. M. Howell and P. W. Codding (1990). Structural requirements for the binding of dexamethasone to nuclear envelopes and plasma membranes. *J Steroid Biochem Mol Biol* **37**(2): 201-14.

Saberi, M., N. B. Woods, C. de Luca, S. Schenk, J. C. Lu, G. Bandyopadhyay, I. M. Verma and J. M. Olefsky (2009). Hematopoietic cell-specific deletion of toll-like receptor 4 ameliorates hepatic and adipose tissue insulin resistance in high-fat-fed mice. *Cell Metab* **10**(5): 419-29.

Sabio, G., M. Das, A. Mora, Z. Zhang, J. Y. Jun, H. J. Ko, T. Barrett, J. K. Kim and R. J. Davis (2008). A stress signaling pathway in adipose tissue regulates hepatic insulin resistance. *Science* **322**(5907): 1539-43.

Sai, S., C. L. Esteves, V. Kelly, Z. Michailidou, K. Anderson, A. P. Coll, Y. Nakagawa, T. Ohzeki, J. R. Seckl and K. E. Chapman (2008). Glucocorticoid regulation of the promoter of 11beta-hydroxysteroid dehydrogenase type 1 is indirect and requires CCAAT/enhancer-binding protein-beta. *Mol Endocrinol* **22**(9): 2049-60.

Saklatvala, J. (2002). Glucocorticoids: do we know how they work? *Arthritis Res* **4**(3): 146-50.

Salojin, K. V., I. B. Owusu, K. A. Millerchip, M. Potter, K. A. Platt and T. Oravecz (2006). Essential role of MAPK phosphatase-1 in the negative control of innate immune responses. *J Immunol* **176**(3): 1899-907.

Sam, S., S. Haffner, M. H. Davidson, R. B. D'Agostino, Sr., S. Feinstein, G. Kondos, A. Perez and T. Mazzone (2008). Relationship of abdominal visceral and subcutaneous adipose tissue with lipoprotein particle number and size in type 2 diabetes. *Diabetes* **57**(8): 2022-7.

Sato, A., H. Suzuki, M. Murakami, Y. Nakazato, Y. Iwaita and T. Saruta (1994). Glucocorticoid increases angiotensin II type 1 receptor and its gene expression. *Hypertension* **23**(1): 25-30.

Schacke, H., W. D. Docke and K. Asadullah (2002). Mechanisms involved in the side effects of glucocorticoids. *Pharmacol Ther* **96**(1): 23-43.

Schoch, G. A., B. D'Arcy, M. Stihle, D. Burger, D. Bar, J. Benz, R. Thoma and A. Ruf (2010). Molecular switch in the glucocorticoid receptor: active and passive antagonist conformations. *J Mol Biol* **395**(3): 568-77.

Seckl, J. R. and B. R. Walker (2001). Minireview: 11beta-hydroxysteroid dehydrogenase type 1- a tissue-specific amplifier of glucocorticoid action. *Endocrinology* **142**(4): 1371-6.

Shang, Y. and M. Brown (2002). Molecular determinants for the tissue specificity of SERMs. *Science* **295**(5564): 2465-8.

Shi, C. S. and J. H. Kehrl (2008). MyD88 and Trif target Beclin 1 to trigger autophagy in macrophages. *J Biol Chem* **283**(48): 33175-82.

Siiteri, P. K. and J. D. Wilson (1974). Testosterone formation and metabolism during male sexual differentiation in the human embryo. *J Clin Endocrinol Metab* **38**(1): 113-25.

Smith, S. S., Q. H. Gong, F. C. Hsu, R. S. Markowitz, J. M. ffrench-Mullen and X. Li (1998). GABA(A) receptor alpha4 subunit suppression prevents withdrawal properties of an endogenous steroid. *Nature* **392**(6679): 926-30.

Smoak, K. A. and J. A. Cidlowski (2004). Mechanisms of glucocorticoid receptor signaling during inflammation. *Mech Ageing Dev* **125**(10-11): 697-706.

Song, I. H. and F. Buttgereit (2006). Non-genomic glucocorticoid effects to provide the basis for new drug developments. *Mol Cell Endocrinol* **246**(1-2): 142-6.

Song, M. J., K. H. Kim, J. M. Yoon and J. B. Kim (2006). Activation of Toll-like receptor 4 is associated with insulin resistance in adipocytes. *Biochem Biophys Res Commun* **346**(3): 739-45.

Stahn, C., M. Lowenberg, D. W. Hommes and F. Buttgereit (2007). Molecular mechanisms of glucocorticoid action and selective glucocorticoid receptor agonists. *Mol Cell Endocrinol* **275**(1-2): 71-8.

Stewart, P. (2002). The adrenal cortex. In: Larsen P, ed. *Williams textbook of endocrinology, Ch 14, 10th ed*(Philadelphia: Saunders): 491-551.

Strissel, K. J., Z. Stancheva, H. Miyoshi, J. W. Perfield, 2nd, J. DeFuria, Z. Jick, A. S. Greenberg and M. S. Obin (2007). Adipocyte death, adipose tissue remodeling, and obesity complications. *Diabetes* **56**(12): 2910-8.

Stromberg, J., T. Backstrom and P. Lundgren (2005). Rapid non-genomic effect of glucocorticoid metabolites and neurosteroids on the gamma-aminobutyric acid-A receptor. *Eur J Neurosci* **21**(8): 2083-8.

Suganami, T., T. Mieda, M. Itoh, Y. Shimoda, Y. Kamei and Y. Ogawa (2007). Attenuation of obesity-induced adipose tissue inflammation in C3H/HeJ mice carrying a Toll-like receptor 4 mutation. *Biochem Biophys Res Commun* **354**(1): 45-9.

Suganami, T. and Y. Ogawa (2010). Adipose tissue macrophages: their role in adipose tissue remodeling. *J Leukoc Biol* **88**(1): 33-9.

Sugiyama, T., D. K. Scott, J. C. Wang and D. K. Granner (1998). Structural requirements of the glucocorticoid and retinoic acid response units in the phosphoenolpyruvate carboxykinase gene promoter. *Mol Endocrinol* **12**(10): 1487-98.

Sunny, N. E., S. Satapati, X. Fu, T. He, R. Mehdibeigi, C. Spring-Robinson, J. Duarte, M. J. Potthoff, J. D. Browning and S. C. Burgess (2010). Progressive adaptation of hepatic ketogenesis in mice fed a high-fat diet. *Am J Physiol Endocrinol Metab* **298**(6): E1226-35.

Takahashi, M., C. Galligan, L. Tessarollo and T. Yoshimura (2009). Monocyte chemoattractant protein-1 (MCP-1), not MCP-3, is the primary chemokine required for monocyte recruitment in mouse peritonitis induced with thioglycollate or zymosan A. *J Immunol* **183**(5): 3463-71.

Takemura, Y., Y. Osuga, T. Yamauchi, M. Kobayashi, M. Harada, T. Hirata, C. Morimoto, Y. Hirota, O. Yoshino, K. Koga, T. Yano, T. Kadowaki and Y. Taketani (2006). Expression of adiponectin receptors and its possible implication in the human endometrium. *Endocrinology* **147**(7): 3203-10.

Thieringer, R., C. B. Le Grand, L. Carbin, T. Q. Cai, B. Wong, S. D. Wright and A. Hermanowski-Vosatka (2001). 11beta-Hydroxysteroid dehydrogenase type 1 is induced in human monocytes upon differentiation to macrophages. *J Immunol* **167**(1): 30-5.

Tomlinson, J. W., J. Moore, M. S. Cooper, I. Bujalska, M. Shahmanesh, C. Burt, A. Strain, M. Hewison and P. M. Stewart (2001). Regulation of expression of 11beta-hydroxysteroid dehydrogenase type 1 in adipose tissue: tissue-specific induction by cytokines. *Endocrinology* **142**(5): 1982-9.

Tomlinson, J. W., E. A. Walker, I. J. Bujalska, N. Draper, G. G. Lavery, M. S. Cooper, M. Hewison and P. M. Stewart (2004). 11beta-Hydroxysteroid dehydrogenase type 1: a tissue-specific regulator of glucocorticoid response. *Endocr Rev* **25**(5): 831-66.

Tsochatzis, E. A., G. V. Papatheodoridis and A. J. Archimandritis (2009). Adipokines in nonalcoholic steatohepatitis: from pathogenesis to implications in diagnosis and therapy. *Mediators Inflamm* **2009**: 831670.

Tuckermann, J. P., A. Kleiman, R. Moriggl, R. Spanbroek, A. Neumann, A. Illing, B. E. Clausen, B. Stride, I. Forster, A. J. Habenicht, H. M. Reichardt, F. Tronche, W. Schmid and G. Schutz (2007). Macrophages and neutrophils are the targets for immune suppression by glucocorticoids in contact allergy. *J Clin Invest* **117**(5): 1381-90.

Tuckermann, J. P., H. M. Reichardt, R. Arribas, K. H. Richter, G. Schutz and P. Angel (1999). The DNA binding-independent function of the glucocorticoid receptor mediates repression of AP-1-dependent genes in skin. *J Cell Biol* **147**(7): 1365-70.

Uysal, K. T., S. M. Wiesbrock, M. W. Marino and G. S. Hotamisligil (1997). Protection from obesity-induced insulin resistance in mice lacking TNF-alpha function. *Nature* **389**(6651): 610-4.

Valenti, L., A. L. Fracanzani, P. Dongiovanni, G. Santorelli, A. Branchi, E. Taioli, G. Fiorelli and S. Fargion (2002). Tumor necrosis factor alpha promoter polymorphisms and insulin resistance in nonalcoholic fatty liver disease. *Gastroenterology* **122**(2): 274-80.

van der Laan, S. and O. C. Meijer (2008). Pharmacology of glucocorticoids: beyond receptors. *Eur J Pharmacol* **585**(2-3): 483-91.

van Loo, G., M. Sze, N. Bougarne, J. Praet, C. Mc Guire, A. Ullrich, G. Haegeman, M. Prinz, R. Beyaert and K. De Bosscher (2010). Antiinflammatory properties of a plant-derived nonsteroidal, dissociated glucocorticoid receptor modulator in experimental autoimmune encephalomyelitis. *Mol Endocrinol* **24**(2): 310-22.

van Schaftingen, E. and I. Gerin (2002). The glucose-6-phosphatase system. *Biochem J* **362**(Pt 3): 513-32.

Vayssiere, B. M., S. Dupont, A. Choquart, F. Petit, T. Garcia, C. Marchandeau, H. Gronemeyer and M. Resche-Rigon (1997). Synthetic glucocorticoids that dissociate transactivation and AP-1 transrepression exhibit antiinflammatory activity in vivo. *Mol Endocrinol* **11**(9): 1245-55.

Wajchenberg, B. L. (2000). Subcutaneous and visceral adipose tissue: their relation to the metabolic syndrome. *Endocr Rev* **21**(6): 697-738.

Wake, D. J., E. Rask, D. E. Livingstone, S. Soderberg, T. Olsson and B. R. Walker (2003). Local and systemic impact of transcriptional up-regulation of 11beta-hydroxysteroid dehydrogenase type 1 in adipose tissue in human obesity. *J Clin Endocrinol Metab* **88**(8): 3983-8.

Walker, B. R. (2007). Extra-adrenal regeneration of glucocorticoids by 11beta-hydroxysteroid dehydrogenase type 1: physiological regulator and pharmacological target for energy partitioning. *Proc Nutr Soc* **66**(1): 1-8.

Walker, B. R. (2007). Glucocorticoids and cardiovascular disease. *Eur J Endocrinol* **157**(5): 545-59.

Walker, B. R. and R. Andrew (2006). Tissue production of cortisol by 11beta-hydroxysteroid dehydrogenase type 1 and metabolic disease. *Ann N Y Acad Sci* **1083**: 165-84.

Walker, B. R. and B. C. Williams (1992). Corticosteroids and vascular tone: mapping the messenger maze. *Clin Sci (Lond)* **82**(6): 597-605.

Wang, D., S. Yamamoto, N. Hijiya, E. N. Benveniste and C. L. Gladson (2000). Transcriptional regulation of the human osteopontin promoter: functional analysis and DNA-protein interactions. *Oncogene* **19**(50): 5801-9.

Wei, L., T. M. MacDonald and B. R. Walker (2004). Taking glucocorticoids by prescription is associated with subsequent cardiovascular disease. *Ann Intern Med* **141**(10): 764-70.

Weisberg, S. P., D. Hunter, R. Huber, J. Lemieux, S. Slaymaker, K. Vaddi, I. Charo, R. L. Leibel and A. W. Ferrante, Jr. (2006). CCR2 modulates inflammatory and metabolic effects of high-fat feeding. *J Clin Invest* **116**(1): 115-24.

Weisberg, S. P., D. McCann, M. Desai, M. Rosenbaum, R. L. Leibel and A. W. Ferrante, Jr. (2003). Obesity is associated with macrophage accumulation in adipose tissue. *J Clin Invest* **112**(12): 1796-808.

White, M. F. (1998). The IRS-signalling system: a network of docking proteins that mediate insulin action. *Mol Cell Biochem* **182**(1-2): 3-11.

White, M. F. (2002). IRS proteins and the common path to diabetes. *Am J Physiol Endocrinol Metab* **283**(3): E413-22.

Whitworth, J. A., G. J. Mangos and J. J. Kelly (2000). Cushing, cortisol, and cardiovascular disease. *Hypertension* **36**(5): 912-6.

Williamson, R. T. (1901). On the Treatment of Glycosuria and Diabetes Mellitus with Sodium Salicylate. *Br Med J* **1**(2100): 760-762.

Windle, R. J., S. A. Wood, N. Shanks, S. L. Lightman and C. D. Ingram (1998). Ultradian rhythm of basal corticosterone release in the female rat: dynamic interaction with the response to acute stress. *Endocrinology* **139**(2): 443-50.

Wissink, S., E. C. van Heerde, B. vand der Burg and P. T. van der Saag (1998). A dual mechanism mediates repression of NF-kappaB activity by glucocorticoids. *Mol Endocrinol* **12**(3): 355-63.

Wust, S., D. Tischner, M. John, J. P. Tuckermann, C. Menzfeld, U. K. Hanisch, J. van den Brandt, F. Luhder and H. M. Reichardt (2009). Therapeutic and adverse effects of a non-steroidal glucocorticoid receptor ligand in a mouse model of multiple sclerosis. *PLoS One* **4**(12): e8202.

Xu, C., J. He, H. Jiang, L. Zu, W. Zhai, S. Pu and G. Xu (2009). Direct effect of glucocorticoids on lipolysis in adipocytes. *Mol Endocrinol* **23**(8): 1161-70.

Xu, H., G. T. Barnes, Q. Yang, G. Tan, D. Yang, C. J. Chou, J. Sole, A. Nichols, J. S. Ross, L. A. Tartaglia and H. Chen (2003). Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *J Clin Invest* **112**(12): 1821-30.

Yamauchi, M., M. Hashimoto, K. Ichiyama, R. Yoshida, T. Hanada, T. Muta, S. Komune, T. Kobayashi and A. Yoshimura (2007). Ifi202, an IFN-inducible candidate gene for lupus susceptibility in NZB/W F1 mice, is a positive regulator for NF-kappaB activation in dendritic cells. *Int Immunol* **19**(8): 935-42.

Yang, C. (2009). Investigation of the cellular and physiological effects of 5α-tetrahydro reduced glucocorticoids in vitro and in vivo., University of Edinburgh. **Thesis for Doctor of Philosophy**.

Yang, H., W. Wei, M. Menconi and P. O. Hasselgren (2007). Dexamethasone-induced protein degradation in cultured myotubes is p300/HAT dependent. *Am J Physiol Regul Integr Comp Physiol* **292**(1): R337-4.

You, M., R. V. Considine, T. C. Leone, D. P. Kelly and D. W. Crabb (2005). Role of adiponectin in the protective action of dietary saturated fat against alcoholic fatty liver in mice. *Hepatology* **42**(3): 568-77.

Yu, J. G., S. Javorschi, A. L. Hevener, Y. T. Kruszynska, R. A. Norman, M. Sinha and J. M. Olefsky (2002). The effect of thiazolidinediones on plasma adiponectin levels in normal, obese, and type 2 diabetic subjects. *Diabetes* **51**(10): 2968-2974.

Yuan, M., N. Konstantopoulos, J. Lee, L. Hansen, Z. W. Li, M. Karin and S. E. Shoelson (2001). Reversal of obesity- and diet-induced insulin resistance with salicylates or targeted disruption of Ikkbeta. *Science* **293**(5535): 1673-7.

Zhang, X. W., Q. Liu, Y. Wang and H. Thorlacius (2001). CXC chemokines, MIP-2 and KC, induce P-selectin-dependent neutrophil rolling and extravascular migration in vivo. *Br J Pharmacol* **133**(3): 413-21.

Zhou, C., S. Verma and B. Blumberg (2009). The steroid and xenobiotic receptor (SXR), beyond xenobiotic metabolism. *Nucl Recept Signal* **7**: e001.

Ziera, T., H. Irlbacher, A. Fromm, C. Latouche, S. M. Krug, M. Fromm, F. Jaisser and S. A. Borden (2009). Cnksr3 is a direct mineralocorticoid receptor target gene and plays a key role in the regulation of the epithelial sodium channel. *FASEB J* **23**(11): 3936-46.

Zierath, J. R., J. N. Livingston, A. Thorne, J. Bolinder, S. Reynisdottir, F. Lonnqvist and P. Arner (1998). Regional difference in insulin inhibition of non-esterified fatty acid release from human adipocytes: relation to insulin receptor phosphorylation and intracellular signalling through the insulin receptor substrate-1 pathway. *Diabetologia* **41**(11): 1343-54.

Zlotnik, A. and O. Yoshie (2000). Chemokines: a new classification system and their role in immunity. *Immunity* **12**(2): 121-7.